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The Composition of Diphtheria Toxin—Antitoxin Floccules.

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The work of Ramon on the flocculation of mixtures of diphtheria toxin and antitoxin has given us a new measure of toxin—the L*f** ; this is defined as the amount of toxin which with a unit of antitoxin flocculates in minimum time. Practically all the toxin and antitoxin in such an indicating mixture are carried down in the precipitate, and we may therefore consider that one L*f* of toxin combines with one unit of antitoxin to form a balanced compound of minimum solubility. From mixtures containing excess of toxin or antitoxin precipitates are formed containing an excess of one of these constitutents, the precipitation is delayed, and when the ratio is altered too far, altogether prevented.

Since practically all the toxin and antitoxin in an indicator mixture are precipitated, a study of the precipitate should shed some light on the nature of antitoxin, and possibly of toxin as well, unless these constituents are hidden among non-specific substances precipitated with them. With this end in view we have applied some new methods, particularly the ultra-violet absorption curve, to the study of the composition of the precipitate, and have investigated the effect of different conditions, particularly the presence of varying concentrations of non-specific proteins, on the amount of precipitate.

* For a discussion of the flocculation method and bibliography, see Bayne-Jones (1928).

Methods.

The antitoxic sera and toxins were kindly supplied to us by Dr. O'Brien, of the Wellcome Physiological Research Laboratories; they were as follows:—

Antitoxic serum A	920 units per 1 c.c.	
" 2737....	910 "	Protein 10·3 per cent.
" 1664....	2730 "	" 15·6 "
" 2902....	17 "	
" 1876....	49 "	*In vivo.*
Cow serum	480 "	"
Antitoxic serum 2885....	About 700 "	
	220 "	Lower zone, " non-specific."
" 293....	40 "	Protein 7·3 per cent.
" Original " pseudoglobulin	1700 "	" 18·5 "
Pseudoglobulin I*	890 "	" 6 "
" II*	177 "	" 2 "
Toxin TX 397	14 units per 1 c.c.	
" TX 422	24 "	

A pseudoglobulin solution (Pseudoglobulin 2737) containing 8·15 per cent. of protein and 910 units per 1 c.c. was made from serum 2737.

Unless otherwise stated the titres of antitoxins and toxins were measured by the flocculation method. The titre in these *in vitro* units does not necessarily agree with the titre in Ehrlich units determined *in vivo* (see Glenny and Wallace, 1925).

To prepare floccules we either heated the mixture of toxin and antitoxin at 46° C. for $1\frac{1}{2}$ hours after flocculation began and kept in the ice chest over night, or froze the mixture solid and left it surrounded by freezing mixture in the ice chest over night, a method of obtaining floccules described to us by Mr. C. G. Pope. The mixture was then centrifuged, the supernatant fluid pipetted off, 0·9 per cent. NaCl solution added, the fluid again centrifuged and the supernatant pipetted off. This washing was repeated twice. Hartley (1926) has shown that only slight reduction of the nitrogen content of the precipitate results from such washing. The resulting precipitate was then dissolved by adding hydrochloric acid to about *p*H 3·0, at which reaction

* These two solutions were prepared by Mr. Pope from " original pseudoglobulin " by electrodialysis. They would not flocculate with toxin and were titrated by blending with antitoxin 2737.

a solution was obtained which was clear by transmitted light but very slightly hazy when viewed against a dark background.

Crude toxin 422 was used in all experiments except I, III and XVII.

Except where otherwise stated we studied the floccules from mixtures of toxin and antitoxin in the proportion of one *Lf* to one unit; such a mixture we have called a balanced mixture. In all cases we have estimated these proportions for the actual toxin and antitoxin under consideration to 5 per cent., except in the case of antitoxic serum 2885, which is mentioned later. The amount of nitrogen found in the floccules is expressed as the amount per unit of antitoxin in the mixture used; except in the unbalanced mixtures this will be the same as the amount per *Lf*.

Nitrogen was estimated either colorimetrically by direct Nesslerisation, using an amount of solution containing some 0·16 mg. N, or by distillation and titration, using amounts containing some 3 mgm. N; the second method was considerably more accurate.

It has been shown by one of us (Smith, 1929) that the method of absorption spectrometry is of value for characterising a protein. This depends on the shape of the absorption curve which is obtained on plotting the values of the extinction coefficient* against a series of wave-lengths. Also, since the amount of absorption is not the same for different kinds of protein, it is necessary to know the nitrogen concentration of the protein solution under test. The protein is then characterised both by the shape of the curve and by the values of the extinction coefficients for a given concentration of nitrogen.

In the same paper (Smith, 1929) it was also pointed out that the ratio $\frac{\text{extinction coefficient at head of curve}}{\text{extinction coefficient at foot of curve}}$ or E_h/E_f could be taken as an index of the purity of the sample. In the case of serum pseudoglobulin, which had been treated with alcohol and ether to remove lipoids, the value of this ratio was 3. Since the toxin-antitoxin floccules used by us were not so extracted, they were compared with pseudoglobulins which were also untreated. The ratio E_h/E of these was about 2·5, the change in value being due to E_f becoming greater, whilst E_h remained the same. All the serum globulins, comprising those of four different species (horse, goat, rabbit and man) so far examined, have shown the same typical absorption curve already described by one of us.

It follows from the above that the method will detect the presence of

* The extinction coefficient $(\log I - \log I')/d$ (where I is intensity of light entering solution and I′ the intensity of that leaving, and d is the thickness of the layer) is directly proportional to the concentration of the substances under test.

nitrogenous impurities in such globulins if they are present in sufficient amount. For example, if 10 per cent. of serum albumin were present in a sample of serum pseudoglobulin it would be detectable, since the extinction coefficient (E_h) of albumin is only about half that of globulin. Again, ammonium salts usually cause but little absorption, and hence if these were present as an impurity the absorption would be small compared with nitrogen content.

In order to be certain that error was not introduced by the presence of nitrogenous substances other than the floccules themselves it was necessary to ensure that they were washed sufficiently with saline before dissolving them. They were usually obtained from mixtures made with crude toxin broth, and this contained nitrogen containing compounds having a very high absorptive power at the wave-lengths with which we were concerned. The curve (3) in fig. 1 shows the absorption curve of the saline used for the first wash of one of our precipitates, about 40 c.c. of saline being used for the process. We found that saline used for the third washing showed no absorption. We therefore washed the precipitate three times in all cases.

The intrinsic error of the method itself is an absolute one of about 0·04 in the value of the extinction coefficient (von Halban and Eisenbrand, 1927). This would correspond to about 4 per cent. of the extinction coefficients with which we were dealing.

The solutions of toxin-antitoxin floccules and of the various pseudoglobulins with which their absorption spectra were compared contained 0·015 per cent. N. The absorption curves were measured with a Hilger quarter-plate spectrometer and rotating sector. The condensed spark between tungsten steel electrodes was used as the source of radiation.

Pseudoglobulin was prepared from antitoxic and normal horse sera by precipitation, thrice, by half-saturation with ammonium sulphate, followed by dialysis to remove salts and precipitate the euglobulin. Whole globulin also was prepared from normal serum, according to the method of Svedberg and Sjögren (1928), except that no toluol was added, bacterial changes being avoided by storing and dialysing in the cold. Hydrogen-ion concentrations were measured with the glass electrode (Kerridge, 1925).

1. *Composition of Floccules.*

Absence of Inorganic Nitrogen.—Flössner and Kutscher (1924) found that 86 per cent. of their floccules consisted of ammonium magnesium phosphate. The presence of such inorganic nitrogen would invalidate both our qualitative and quantitative conclusions. Actually we have found that the nitrogen in

our floccule solutions was wholly precipitated by tungstic acid, so that no such simple nitrogen compounds can have been present. Crystals of ammonium magnesium phosphate settled at the bottom of our bottles of toxin, but we excluded them from the samples that we used for flocculation.

Absorption Spectrum. A description of the method and its possible errors has been given above. It was thought that the various experimental conditions under which the absorption was measured might introduce further error. The following controls were made :—

(1) The absorption of normal horse-serum globulin was measured at *p*H 3 and *p*H 6 ; also after heating for $1\frac{1}{2}$ hours at 46° C. at *p*H 6. No differences in the absorption curve were noticed.

(2) The pseudo-globulin fraction of an antitoxic serum was measured under the same conditions as (1) above. The absorption curve was the same as for normal pseudo-globulin in each case.

Fig. 1 shows an absorption curve for a toxin—antitoxin floccule solution (No. XXII, Table III) the nitrogen content of which was measured by the titration method. This curve is practically identical with that of pseudo-globulin. Four other floccule solutions (XVIII, XX, XXII, XL), the nitrogen content of which was estimated by the titration method, gave identical absorption curves, except that the value for E_f was slightly higher, probably owing to a slight haziness which would cause scattering at the lower wave-length.

In 25 other floccule solutions the nitrogen content estimated from the absorption curve, using the value of E_h found with the above five solutions, was compared with the nitrogen content measured by the less accurate colorimetric Kjeldahl method. The average difference between the results of the two methods was 1·8 per cent. ; the difference was within 10 per cent. in all but two, and within 5 per cent. in all but five. This agreement is as good as could be expected, if allowance is made for the possible errors of the two methods ; with improvements of the technique of the colorimetric estimations in the later experiments the discrepancies were reduced below 5 per cent.

As the spectrometric method was the more trustworthy we have used the results given by it in subsequent work.

Colour with Folin's Phenol Reagent.—The colour given by Folin's phenol reagent has been used with some success for estimating proteins. The amount of colour given apparently depends on phenol groups present in the molecule in some special state, not on the total phenol groups contained, since the colour given by a protein is less than that given by the tyrosin and tryptophane it

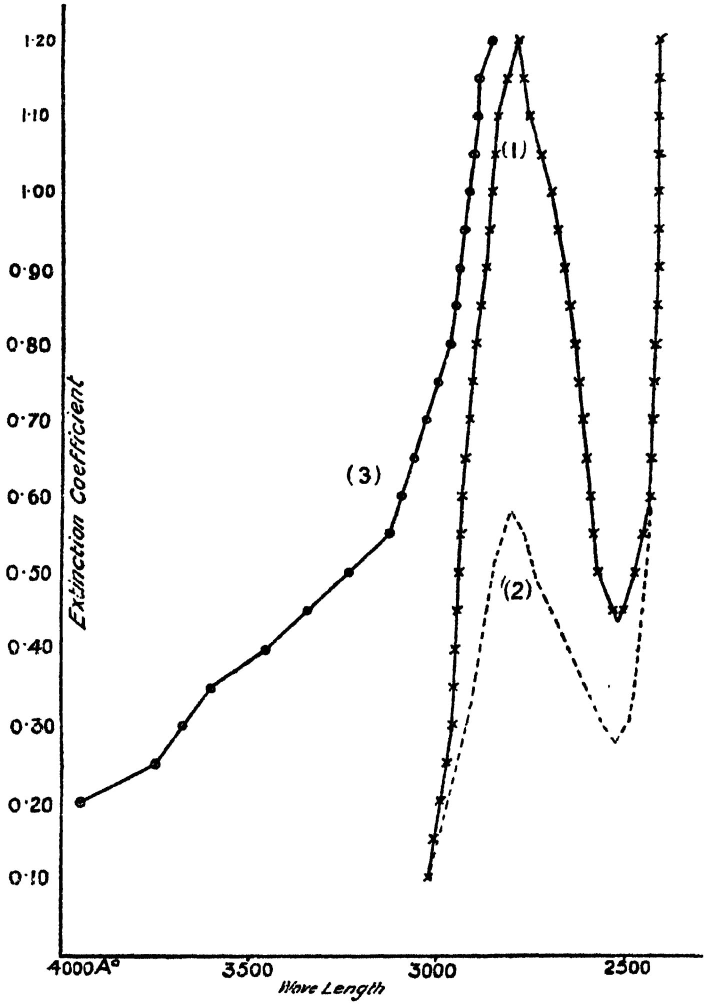

FIG. 1.—Ultra-violet absorption curves.

(1) { Floccule solution containing 0·0155 gm. N per 100 c.c.
Pseudo-globulin solution containing 0·0155 gm. N per 100 c.c.

(2) Serum albumin ,, ,, ,, ,,

(3) Washings from floccules ,, ,, ,,

contains. Using the method described by Greenberg (1929) we found that the same depth of colour was given with this reagent by the floccules as was given by pseudoglobulin. Floccules were prepared by freezing from a balanced mixture of toxin 422 and pseudoglobulin from antitoxic serum 1664, which were washed and dissolved in the usual way; this solution was compared with a 0·097 per cent. solution of the same pseudoglobulin. Measured by the

extinction coefficient the floccule solution contained 0·0136 per cent. of nitrogen; by the phenol reagent it contained 0·0133 per cent. of nitrogen. The floccules therefore give the same amount of colour per gram of nitrogen with this reagent as does pseudoglobulin.

It might be supposed that this colorimetric method measured the same property of proteins as the ultra-violet absorption, since both depend on phenol groups. This, however, is not the case, for serum albumin, which gives about the same amount of colour per gram of nitrogen as globulin, has only half the absorptive power.

Lipoid.—We have found only very small traces of ether or chloroform soluble substances in our floccules (Experiments XXI and XXIA). Flössner and Kutscher (1924), however, found 18·5 per cent. of the organic matter in their floccules to be soluble in alcohol and ether, and of this two-thirds was soluble in chloroform. This point is important in view of the significance attached to lipoids in immunity reactions, and particularly the fact that Hartley (1925, 2) found that diphtheria antitoxin extracted with alcohol and ether would not flocculate with toxin, although it still retained its protective power for animals. Flössner and Kutscher washed their floccules with distilled water, in which the floccules disperse, and it seems to us probable that they washed away the greater part of the specific precipitate. If the toxin they used was of moderate strength, they should have got about 0·13 gm. of organic nitrogen in their floccules; this would make the ratio lipoid/organic N equal to 0·035/0·13, which is of the same order as that found by us in Experiment XXIA. It is very possible that these small quantities are accidental contaminations which cannot be removed by washing with salt solution.

Experiment XXI.—250 c.c. of toxin 422 were mixed with 3·5 c.c. of "original pseudoglobulin" and frozen. Next day the floccules were centrifuged off, washed three times with saline, suspended in distilled water and brought to pH 3·0 with 0·04 N . HCl. The resulting clear solution was made up to 12 c.c. To 8·5 c.c. of this were added 35 c.c. of absolute alcohol. The precipitate was centrifuged down and the supernatant pipetted off, 80 c.c. of absolute alcohol were added, the precipitate again centrifuged down and the supernatant pipetted off; this was repeated. The final precipitate was dried *in vacuo*, and extracted with hot chloroform; the chloroform was evaporated down and no detectable residue obtained. From the supernatant alcohol washings 0·3 mgm. of ether-soluble material was obtained; dissolved in chloroform this material gave with acetic anhydride and sulphuric acid a very doubtful greenish colour, very faint compared with that given by 0·016 mgm. of cholesterol.

The total nitrogen in the precipitate was 10·8 mgm., corresponding to 69·3 mgm. of protein, so that the alcohol and ether-soluble substances were less than 1 per cent. of the whole. The smallness of the amount of lipoid found might have been due to the use of purified pseudoglobulin as antitoxin.

Experiment XXIa.—The intention in this experiment was to provide more lipoid for precipitation. To 100 c.c. of toxin 422 were added 2·4 c.c. of antitoxic serum A.T. 2737, 25 c.c. of normal horse serum containing 0·095 per cent. of cholesterol, and 15 c.c. of human serum containing 0·20 per cent. of cholesterol. The mixture was frozen and washed as before, suspended in 1·5 c.c. of distilled water and added gradually to 60 c.c. of a mixture of alcohol 3 parts and ether 1 part, as in the method of Bloor, Pelkan, and Allen (1922) for estimating cholesterol in blood. The mixture was boiled, cooled, made up to 100 c.c. with alcohol-ether mixture and filtered; 80 c.c. of the filtrate were evaporated and the residue extracted with hot chloroform; this on evaporation gave 0·6 mgm. of residue, which on redissolving in chloroform gave with acetic anhydride and sulphuric acid a very doubtful greenish colour, much less than that given by 0·016 mgm. of cholesterol.

The nitrogen in these floccules should correspond to about 25 mgm. of protein, so that the chloroform soluble part did not exceed 3 per cent. of the total precipitate.

Titration.—Fig. 2 shows the titration curves of three batches of floccules and two specimens of pseudoglobulin. These floccules were washed with boiled

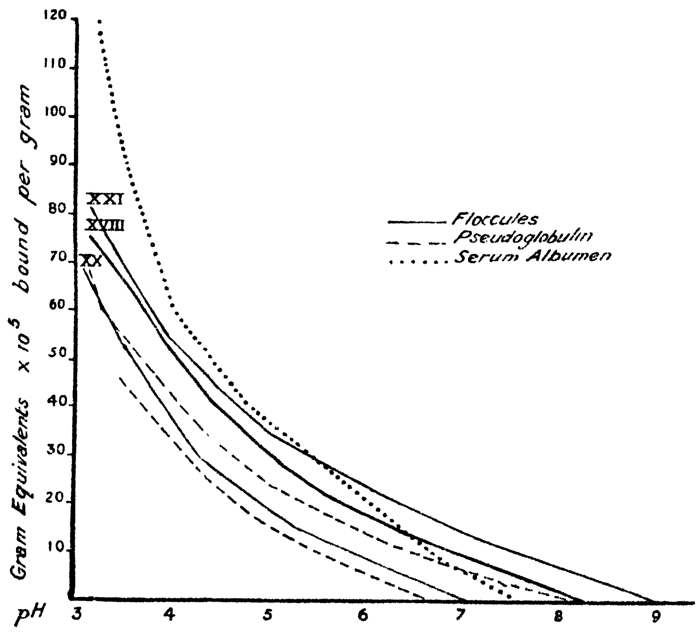

Fig. 2.—Titration curves.

0·9 per cent. saline and shaken up with boiled distilled water before titration with 0·04 N. HCl. The globulins had been dialysed against freshly distilled water, out of contact with air, and were diluted with boiled distilled water; sodium chloride solution was also added, approximately equal to the amount which could not be pipetted off from the floccules after centrifuging. Quantities of globulin and of floccules containing approximately the same amount of

nitrogen were used. To obtain the amount of HCl combined, the free HCl calculated from the *c*H, using the activity coefficients of Scatchard (1925), was subtracted from the total HCl added. The figures below *p*H 3·5 are unreliable, since the free HCl becomes of the same order as the combined at this level and small errors in *p*H measurements and in the activity coefficients used make a large difference in the result.

The *p*H at which the curves start varies owing to the varying degree to which base was removed in washing the floccules. If the acid-binding powers of the floccules and globulins were the same, the vertical distances between the curves should remain the same throughout their length. Actually the floccule curves are steeper, that is the acid-combining power of the floccules is higher; but the difference is slight, as is shown by comparison of the curve for serum albumen.

Re-dispersion of Floccules.—It was found that if the floccules obtained by freezing were washed with distilled water and centrifuged down, and the deposit so obtained shaken up with more distilled water, it dispersed completely, giving a solution clear by transmitted light but slightly hazy when viewed against a dark background. Salt solutions were added to samples of this solution to bring about the concentrations shown in Table I. The results show that this "solution" is reprecipitated like a negatively charged hydrophobe colloid according to Hardy's rule. As the *p*H of the solution is between 7 and 8 the behaviour is what would be expected from a denatured globulin.

Table I.—Molar Concentrations of Salts required to produce opacity and flocculation of Toxin-Antitoxin floccules dispersed in Distilled Water.

Salt.	Opacity.	Floccules.
$NaCl$	0·0075	0·03
Na_2SO_4	0·01	0·05
$CaCl_2$	0·0005	0·00125
$Ce(Ac)_3$	0·00006	0·0003

It was noted by Madsen and Schmidt (1926) that high concentrations of salts delayed or checked the flocculation of toxin and antitoxin, and that the effect of halogen anions was in the order I > Br > Cl. With floccules which had been left at 46° C. for ½ hour after formation and then centrifuged down, freed from supernatant fluid and tested at once, we obtained the results shown in Table II. The anions appeared to follow the order I > SCN > Br > Ac > NO_3 while there was no appreciable difference between the salts of different kations with the same anion.

Table II.—Dispersion of Toxin-Antitoxin floccules in concentrated Salt Solutions.

	Li.	Na.	NH_4.	K.	Rb.	Cs.
NO_3	—	—	2 N§	2 N§	—	—
Br	—	—	—	4 N†	—	—
				2 N‡	—	—
CH_3COO	—	2 N†	4 N‡	4 N‡	—	—
		1 N‡	2 N‡	2 N‡	—	—
SCN	—	2 N*	2 N*	2 N*	—	—
		1 N†	1 N‡	1 N‡	—	—
I	1 N†	2 N*	2 N*	2 N*	—	—
		1 N†	1 N†	1 N†	1 N†	1 N†
Salicylate	1 N†	1 N*	—	—	—	—
	0·5 N‡	0·5 N‡	—	—	—	—

* Dispersed completely.
† Dispersed incompletely.
‡ Amount of precipitate reduced.
§ Amount of precipitate unaffected.

This effect of strong salt solutions resembles that on serum albumin coagulated by heat, described by Wilheim (1929).

As a result of these investigations we can say that the floccules consist mainly of a protein which has the properties of a denatured pseudoglobulin, and in absence of evidence to the contrary we feel justified in concluding that it actually is pseudoglobulin.

II.—*Quantitative.*

Non-specific proteins may be carried down with the antigen and antibody in the floccules ; but it is probable that protein, which did not actually combine with the toxin, would not be denatured, and would be removed on washing. If, however, an appreciable amount resisted removal, it would be expected that this amount of absorbed protein would vary with the conditions under which flocculation took place, and, particularly, would be increased by the addition of non-specific proteins to the flocculating mixture. We have therefore measured the amount of protein in the floccules found under varying conditions.

Constancy of Results.—Seven pairs of estimations were made on the mixtures of toxin and antitoxin treated in the same way but at different times (Table III). The agreement between these pairs was good, except in the pair XXVIII and XXXII, in which the discrepancy between the two results (14 per cent.) was not excessive for a process of this kind ; some discrepancies may be expected from losses during washing.

Table III.—Duplicate Estimations.

Experiment No.	Antitoxin.	Process.	Floccules (mgm. N per unit × 10^4). Spectro-metric.	Floccules (mgm. N per unit × 10^4). Kjeldahl.	
I	A	Heat	16·7	—	Toxin TX 397 used.
III			16·0	—	
VIII	Pseudo 2737	Heat	15·6	15·7	
X			15·6	14·3	
XXI	"Original pseudo-globulin"	Frozen	18·7	18·0	
XXII			18·0	17·7	
XXIII	,,	Heat	17·4	16·3	
L			18·1	—	
XXVIII	2737	Frozen	15·4	15·0	
XXXII			17·6	17·6	
XXXIV	1664	Heat	15·0	—	
XLVII			15·4	—	
IX	Pseudo 2737 in excess	Heat	13·8	—	
XIII			13·0	13·5	

Effect of Heat or Freezing (Table IV).—There was little difference in the amount of precipitate obtained by the two methods when the toxin and antitoxin are nearly balanced. In the unbalanced mixtures more was obtained by the freezing method; probably because flocculation is slower in unbalanced mixtures and is not complete in the time allowed in the heating process.

Table IV.—Comparison of Heating and Freezing Methods.

Experiment No.	Antitoxin.	Process.	Floccules (mgm. N per unit × 10^4). Spectro-metric.	Floccules (mgm. N per unit × 10^4). Kjeldahl.
VIII	Pseudo 2737	Heat	15·6	15·7
XVIII		Frozen	15·7	15·4
XII	Pseudo 2737, toxin in excess	Heat	130·	11·3
XV		Frozen	15·0	15·3
XIII	Pseudo 2737, anti-toxin in excess	Heat	13·0	13·5
XVI		Frozen	14·1	13·5
XXIII	"Original pseudo-globulin"	Heat	17·4	16·3
L			18·1	—
XXI		Frozen	18·7	18·0
XXII			18·0	17·7
XIV	2737	Heat	16·2	17·4
XXVIII		Frozen	15·4	15·0
XXXII			17·6	17·6

Effect of Dilution and Salt Concentration (Table V).—In the process of freezing the toxin-antitoxin mixtures, the toxin, antitoxin and electrolytes are greatly concentrated in the unfrozen liquid, owing to the separation of water crystals (see Freundlich, 1922). Since the amount of precipitate obtained by freezing was approximately the same as that obtained by heating it is probable that the amount is independent of the concentration of the mixture and of the electrolytes in it. This is borne out by experiments LI and XLVIII. The chloride concentration in the toxin was only 0·019 N and the conductivity equal to that of a 0·033 N sodium chloride solution, so that the electrolyte concentration was at the lower level of that found necessary to bring about reprecipitation of the dispersed floccules (Table I), but an increase of the electrolyte concentration by 0·2 N did not affect the amount of precipitate.

Table V.—Effect of Dilution, Salt Concentration and Reaction.

Experiment No.	Antitoxin.	Process.	Floccules (mgm. N per unit × 10⁴). Spectrometric.	Kjeldahl.	
XXIII	"Original pseudo-globulin"	Heat	17·4	16·3	Undiluted.
L			18·1	...	Undiluted.
LI			18·1	—	Toxin diluted with an equal volume of broth.
XXXIV	1664	Heat	15·0	15·0	Approximately 0·033 normal salt.
XLVII			15·4	—	
XLVIII			15·4	—	2·75 c.c. of 2N . NaCl solution added to 25 c.c. of toxin.
XXVIII	2737	Frozen	15·4	15·0	*p*H of mixture 8·3.
XXXII			17·6	17·6	*p*H of mixture 8·3.
XXXIII			17·4	17·1	*p*H of mixture 7·07.
XX			...	17·7	*p*H of mixture 6·5; nitrogen estimated by titration method.

Effect of pH (Table V).—Changes of *p*H from 6·4 to 8·4 make no difference to the titration of toxin by flocculation (Bayne-Jones, 1928). Experiments XXVIII, XXXII, XXXIII, XX show that *p*H variations within this range made no difference to the amount of precipitate obtained.

Addition of Non-specific Protein (Table VI).—It is generally supposed that the amount of precipitate in an immunity reaction is increased by the addition of non-specific proteins. However, we have found no difference in the amount of precipitate per unit antitoxin when pseudoglobulin prepared from serum

was used instead of the original serum; nor on the other hand when normal horse serum, or pseudoglobulin prepared from normal horse serum, was added at the time of mixing the toxin and antitoxin, although the total protein in the mixture was thereby increased to six times and the pseudoglobulin to four times that present when the antitoxic pseudoglobulin was used alone. Nor was there any appreciable increase of the protein in the precipitate when fresh guinea-pig serum was added at the time of mixing, although absorption of complement was shown to have taken place.

Experiment XXXI. Absorption of Complement.—15 c.c. of toxin 422 were mixed with 1·2 c.c. of fresh guinea-pig serum. Decreasing quantities of this mixture were added to 0·5 c.c. of sensitised 5 per cent. sheep corpuscles suspension, and the volume made up to 2 c.c. with 0·9 per cent. saline. Hæmolysis was complete in 20 minutes in the tube containing 0·3 c.c. of the mixture, but incomplete in the tube containing 0·2 c.c.; so that hæmolysis was complete with the complement diluted 1/83; without the toxin hæmolysis occurred with the complement diluted 1/240.

To 13·5 c.c. of the mixture of toxin and guinea-pig serum were added 0·3 c.c. of antitoxin 2737 to form a balanced mixture; this was frozen solid and kept in the ice chest overnight. Next day the floccules were separated and treated as usual, and the supernatant fluid tested for complement. Decreasing quantities from 1·5 c.c. were mixed with sensitised corpuscles as before; no hæmolysis occurred in any of the tubes in 1 hour.

Table VI.—Effect of Non-specific Proteins.

Experiment No.	Antitoxin.	Process.	Floccules (mgm. N per unit · 10^4). Spectrometric.	Floccules (mgm. N per unit · 10^4). Kjeldahl.	
XIV	2737 }	Heat	16·2	17·4	
VIII	Pseudo 2737 }		15·6	15·7	
XXVIII	2737 alone	Frozen	15·4	15·0 }	Antitoxin contained 0·113 mgm. protein per unit.
XXXII	,,	,,	17·6	17·6 }	
XVIII	Pseudo 2737 alone	,,	15·7	15·7	0·090 mgm. protein per unit
XXX	2737 + normal pseudoglobulin	,,	16·3	16·3	0·22 mgm. of normal pseudoglobulin added per unit.
XXXI	2737 + guinea pig serum	,,	—	16·5	0·0033 c.c. of guinea-pig serum added per unit.
XXIII }	"Original pseudoglobulin" alone	Heat	17·4	16·3	Antitoxin contained 0·109 mgm. protein per unit.
L }			18·1	—	
XXIV	"Original pseudoglobulin" + normal pseudoglobulin	,,	17·9	18·3	0·23 mgm. of normal pseudoglobulin added per unit.
XXV	"Original pseudoglobulin" + normal serum	,,	17·1	16·8	0·0083 c.c. of normal serum = 0·58 mgm. of protein, added per unit.
XVII	2737	Frozen	—	19·0	Purified toxin used.

Purified Toxin.—In experiment XVII toxin containing 10×10^{-4} mgm. N per L_f, prepared from toxin 422 by precipitation at *p*H 5·2 and solution in alkali, was used; this preparation should be free from bacterial proteins other than toxin (Locke and Main, 1928); the solution contained 350 L_f in 1 c.c. The floccules obtained were redissolved with acid, but did not form a sufficiently clear solution for spectrophotometry to be possible. From this experiment it appears that the non-toxic constituents of crude toxin do not add to the floccule nitrogen, when an antitoxic serum of high titre is used.

This experiment gave a striking example of a non-specific substance which is not absorbed by the floccules. In attempts to purify toxin it is extremely difficult to remove a red pigment which is precipitated with it; the purified toxin used in this experiment was bright pink, but the floccules obtained from it were white and formed a colourless solution. This pigment is also present when crude toxin is used, but the way in which the toxin is freed from it in the process of flocculation is then less obvious.

These quantitative experiments indicate that the amount of protein per unit of antitoxin in the floccules obtained from balanced mixtures is constant for a given serum or the pseudoglobulin prepared from that serum.

Ratio of Antitoxin to Toxin (Table VII).—We have so far considered only the amount of precipitate obtained from balanced mixtures of toxin and antitoxin. Table VII shows that when the ratio of antitoxin to toxin is increased the amount of precipitate per L_f is increased while the amount of precipitate per unit of antitoxin is maximum when the ratio is 1·0 but remains near this level from ratio 0·75 to 1·5. Flocculation could not be obtained when the ratio was below 0·6 or above 1·6. These results are very similar to those found

Table VII.—Effect of Ratio Antitoxin/Toxin.

Experiment No.	Antitoxin.	Process.	Floccules (mgm. N per unit × 10^4).		Floccules (mgm. N per L_f × 10^4).		
			Spectro-metric.	Kjeldahl.	Spectro-metric.	Kjeldahl.	
XXXVII	2737	Frozen	1·87	1·77	1·12	1·06	Ratio = 0·60.
XV	Pseudo 2737	,,	15·0	15·3	11·3	11·5	Ratio = 0·75.
XVIII	2737	,,	15·7	15·4	15·7	15·4	Ratio = 1·00. Balanced
XVIII	2737	,,	14·4	14·0	18·7	18·2	Ratio = 1·30.
XVI	Pseudo 2737	,,	14·1	13·5	21·1	20·3	Ratio = 1·50.

with a precipitin reaction by Dean and Webb (1926), although the range over which they found flocculation was much wider. The spectroscopic results agree with the nitrogen estimations as well in the unbalanced as in the balanced floccules.

Amount of Precipitate with Different Antitoxins (Table VIII).—The first five antitoxins were sera of high titre or pseudoglobulins prepared from these; the agreement is very close except for XXIII, and in this case the difference is hardly significant when the possible differences between amounts of precipitate found with the same toxin, and the possible errors in standardising the antitoxin, are taken into account. Hartley (1925, 1926) with similar antitoxins obtained from $11 \cdot 4 \times 10^{-4}$ to $24 \cdot 9 \times 10^{-4}$ mgm. of floccule nitrogen per unit; it is possible that, as his mixtures stood much longer than ours, he may have had some ammonia nitrogen in his floccules which would account for the higher figures; apart from this it is possible that the titres of the sera employed by Hartley were given in *in vivo* units and are therefore not strictly comparable with ours; if this was the case the agreement between his results and ours is as good as could be expected.

Table VIII.—Different Antitoxic Sera.

Experiment No.	Antitoxin.	Process.	Floccules (mgm. N per unit $\times 10^4$).		Floccules (mgm. N per $L_f \times 10^4$).		
			Spectrometric.	Kjeldahl.	Spectrometric.	Kjeldahl.	
III	A	Heat	16·0	15·2	16·0	15·2	
XIV	2737	,,	16·2	17·4	16·2	17·4	
XXXV	2737, 150 units with pseudoglobulin I, 450 units	,,	15·7	15·7	15·7	15·7	
XXXVI	2737, 150 units with pseudoglobulin II, 450 units	,,	15·5	15·5	15·5	15·5	
XXXIV	1664	,,	15·0	15·0	15·0	15·0	
XXIII	"Original pseudoglobulin"	,,	17·4	17·4	17·4	17·4	
XLI	2885, 0·75 c.c. to 600 L_f	Frozen	17·0	17·0	17·0	17·0	Mgm. N per unit doubtful.
XLII	2885, 1·12 c.c. to 600 L_f	,,	—	—	17·6	18·3	
XXXIX	2885, 2·7 c.c. to 600 L_f	,,	—	—	32	32	
XLIII	2931	Heat	—	—	31·0	31·5	
XLII	2902	,,	—	—	—	25	
XXXVIII	1876	,,	—	—	—	50·5	

Serum 2885 was peculiar; not only did it give two zones, but also in the upper zone we were unable to select the indicator mixture, as several tubes flocculated simultaneously while others between remained clear, as though there were several sub-zones in this upper zone. We therefore measured the amount of floccules with quantities of antitoxin corresponding to 533 and to 800 units per 1 c.c., values calculated from the extremes of this doubtful zone. We cannot calculate the floccule N per unit as we are uncertain of the titre of the serum; the floccule nitrogen per L_f is but little higher than that found with the first four sera. This serum flocculated very slowly, taking 4 hours at 46° C. with toxin 422, while the previous sera flocculated in about 25 minutes. Glenny and Wallace (1925) were of the opinion that slowly flocculating sera give larger precipitates; this was not borne out in this case.

In the lower zone of serum 2885, which if it were a toxin-antitoxin flocculation would correspond to 220 units per 1 c.c. of serum, the floccules contained 32×10^{-4} mgm. N per L_f of toxin added. This was a "non-specific" zone, that is a zone in which a reaction is taking place between a bacterial protein other than toxin and its antibody; the antigen and antibody were not those with which we are dealing and the results are of no significance.

Serum 2931, which had a very low titre, also gave high floccule nitrogen. In this case the flocculation was specific: when the serum was blended with another of high titre known to be specific, the titre of the mixture was found to be equal to the sum of the titres of the components, showing that serum 2931 and the high titre serum reacted with the same antigen. However, it is possible that antibodies to bacterial proteins other than toxin were present in this and in other sera in small quantities. With the high titre sera such antibodies would have no effect on the precipitate, as their amount would be too small in relation to the antitoxin: but when the antitoxin titre was low, as with serum 2931, they might be present in sufficient concentration to increase the total precipitate; this also applies to serum 2962.

The value of experiment XXXVIII is doubtful as the serum was contaminated and smelt decomposed, but we got the usual amount of floccule with another serum 1664 when it was contaminated; the precipitate in experiment XXXVIII, however, is probably "non-specific."

It is possible that the simultaneous precipitation with bacterial proteins other than toxin is not the correct explanation of the larger amount of floccule nitrogen per unit antitoxin and per L_f with sera 2931 and 2901; for Dean and Webb (1926) found a larger amount of precipitate per unit of antigen from antisera of low titre than from those of high titre. But here also there may be

two substances precipitating, since they used foreign serum as their antigen and it is possible that the antisera of low titre precipitated both albumin and globulin, while those of high titre precipitated only globulin.

Our previous experiments (Table VI) dispose of the possibility that the larger amount of floccule nitrogen is due to the larger amount of non-specific serum protein present.

Discussion.

We conclude from the investigations in the first part of this paper that the floccules consist almost entirely of denatured pseudoglobulin. It is probable that the toxin contributes only a small fraction of the floccules. The work of Welsh and Chapman (1906) indicated that the greater part of the precipitate in an immunity reaction came from the antiserum. Wu, Chang, and Li (1928), using hæmoglobin as antigen, found that the antigen formed only one-tenth of the precipitate ; and Dean and Webb (1926), using foreign serum as antigen, found very similar proportions when the conditions were such that all the antigen was precipitated. Diffusion experiments indicate that toxin molecules are only one-tenth the size of antitoxin molecules (Arrhenius, 1908) : according to Locke and Main (1928) toxin can be prepared containing only 8×10^{-4} mgm. N per L_f, and there is no reason to suppose that such a preparation, contaminated as it certainly is with pigment, is pure. It is quite conceivable that the very active antigen, diphtheria toxin, differs from such poor antigens as hæmoglobin and serum proteins in having a large part of its relatively small molecule made up of antibody binding groups, so that a much smaller amount is required to precipitate an antibody molecule and that the antigen forms a much smaller proportion of the precipitate. The floccules therefore probably consist mainly of pseudoglobulin derived from the antiserum.

This pseudoglobulin might be made up mainly of non-specific proteins carried down with the specific substances. However we have found in the second part of this paper that the pseudoglobulin flocculated per unit of antitoxin of a given serum, when mixed with an equivalent amount of toxin, is independent of the conditions under which flocculation took place, and that there was no evidence that non-specific proteins were carried down with the floccules. We conclude that this constant amount of pseudoglobulin is related to the toxin-binding properties of the serum in a special way. Although it is possible that antitoxin may exist free from protein, it seems more reasonable to suppose that this pseudoglobulin is the actual antitoxin, and differs from normal pseudoglobulin in the structure of its molecule. That such differences of structure, without differences in the ordinary physical and chemical properties,

can occur, is shown by the immunological reactions of the pseudoglobulins of difference species.

If this supposition is correct we can deduce from our results a standard of purity of antitoxin, viz., about 15×10^{-4} mgm. N per unit, which is of about the same order as that of the preparations of Ramon (1923) and Locke and Main (1926).

If we do not consider that the larger amounts of floccule N per unit found with the sera of low titre are due to simultaneous precipitation from a bacterial protein-precipitin reaction, we must suppose that the number of toxin-binding groups per antitoxic globulin molecule may vary in different sera, and possibly in the same serum, so that our figure is only an average. In the process of immunisation an increase of serum globulin occurs, much greater than the antibody formed will account for, supposing the excess were antitoxin. It is possible that globulins with varying numbers of toxin-binding groups are formed, and that animals which immunise badly differ from those that form potent sera, not in producing fewer antitoxic globulin molecules, but in forming new molecules with few or no binding groups; not, that is, in the quantity but in the quality of the antitoxic globulin they produce.

Since the same results are obtained when toxin-antitoxin mixtures are frozen as when they are heated to 46° C. we cannot consider that the combination of toxin and antitoxin depends on the state of aggregation of the antitoxic globulin. Unpublished experiments of Marrack and Hewitt on osmotic pressures show that globulins are more aggregated at lower temperatures, this increased aggregation accounting for the increased viscosity with falling temperature found by Chick (1914).

Summary.

1. The floccules formed by diphtheria toxin and antitoxin closely resemble serum pseudoglobulin.

2. The ultra-violet absorption curves of the floccules and pseudoglobulin are identical.

3. The amount of precipitate obtained from a balanced mixture of a given toxin and antitoxin is to a large extent independent of the conditions under which flocculation takes place, particularly of the amount of non-specific protein present.

4. Lipoid material forms a very small fraction of the floccules.

5. We consider that the floccules consist mainly of antitoxin, and infer that

antitoxin is not merely carried down in the pseudoglobulin fraction but actually is pseudoglobulin.

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Cellular Individuality in the Higher Animals, with Special Reference to the Individuality of the Red Blood Corpuscle.

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CONTENTS.

Introduction.

For the investigation of cellular individuality in the higher animals the red blood corpuscle naturally suggests itself, for many reasons, as the cell most suitable for experimental study, and it was with this cell that Bordet (1898) carried out his pioneer work in the application of the methods of immunity to the study of the specificity of the animal cell. He showed that the immunisation of an animal with the red cells from an animal of a different species leads to the formation of antibodies (hæmolysins and hæmagglutinins) in the blood of the immunised animal, and that these antibodies are to a large extent specific, in that they act much more powerfully on the red blood corpuscles of animals of the species whose blood has been used for the immunisation than on those of other species—thus showing that the red cell possesses a marked and easily demonstrable "species specificity."

A definite advance was the discovery by Ehrlich and Morgenroth (1900)

that it was not necessary, for the formation of antibodies, that the injected corpuscles should be from an animal of a different species. By the injection of goats with the red blood cells of other goats, they were able to prepare in the goat hæmolysins for goat's corpuscles. Such antibodies, formed as a consequence of the injection of cells of the same species, they termed "isolysins," in contradistinction to the "heterolysins" formed after the injection of foreign cells.

Ehrlich and Morgenroth found that the injection of goat's blood into other goats did not always give rise to the formation of a hæmolysin, and in a number of cases they obtained quite negative results. They were, however, able in 13 goats to prepare isolytic sera, and from a careful study of these concluded that they all differed from one another, *i.e.*, that they all represented different isolyins.

The investigation of the isolysins was continued by Todd and White (1910[1], 1910[2], 1911) who, working in Egypt, had at their disposal 106 cattle which, in the course of immunisation for the production of cattle-plague serum, had been injected with large quantities of the blood of other cattle. The sera of these immune animals at first appeared to be practically without action on bovine corpuscles, but further enquiry showed that this apparent inactivity was due to the absence of a suitable complement, and that, when this was provided in the form of fresh guinea pig serum, 76 out of the 106 sera were very actively isolytic, thus providing a wealth of material for study.

A detailed examination showed that, when one of these active sera was tested on a series of corpuscles, its hæmolytic power was quantitatively very different for the corpuscles of different individuals; being very high for some, less so for others, and again comparatively low for others. A second active serum, tested on the corpuscles of the same individuals, showed similar variations, but these did not coincide with those obtained with the first: that is to say, corpuscles which were highly hæmolysed by the first serum were often comparatively slightly affected by the second, and *vice versa*. When the tests were extended to other sera similar results were obtained—in short no two sera appeared to be absolutely alike.

It was next found that if one of these isolytic sera is "exhausted" with the corpuscles of a particular individual, Ox (A), by treatment with excess of the cells, it remains hæmolytic for the corpuscles of many other individuals, but loses its hæmolytic power for the corpuscles of some other individuals, as well as for those of A. If now a second isolytic serum is exhausted with the corpuscles of the same individual (A) and then tested, its action on the various

corpuscles is not exactly parallel to that of the first exhausted serum, and often shows marked differences. This result was to be expected, as it was shown by Ehrlich and Morgenroth that two goats, each injected with similar doses of the same goat's blood at the same time, gave different isolysins.

There can in fact be no doubt that the character of the isolysins formed on the injection of animals with the corpuscles of animals of the same species depends upon two distinct factors :—

(*a*) The individuality of the injected corpuscles.

(*b*) The individuality of the animal into which they are injected.

A consideration of the enormous number of variations possible in each of these factors shows the almost unlimited possibilities of variation in the resulting sera.

In view of the above it seemed that it should be possible, by taking a mixture of a sufficiently large number of these immune sera, and exhausting it with the corpuscles of one individual, to obtain a serum which would have no hæmolytic action on these corpuscles, but would still hæmolyse those of all other individuals of the same species, and which might therefore be used for the identification of this particular individual.

To test this possibility a very highly polyvalent serum, prepared by mixing between 60 and 70 of the isolytic sera, was exhausted with the corpuscles of one particular individual and then tested on the red blood cells of 110 other cattle. It was found that the exhausted serum, while naturally without action on the corpuscles of the animal for which it had been exhausted, was still powerfully hæmolytic for the corpuscles of every one of the 110 other cattle ; so that it could be regarded as then constituting a specific reagent for the corpuscles of an individual ox.

This test was repeated a number of times with the cells of different individuals, and similar results were constantly obtained, except in the case of close blood relations, where exceptions may occur. These will be referred to later.

A method being thus available of identifying the corpuscles of a certain individual, even in the presence of corpuscles of other animals of the same species, it was possible to study the question of what happens when the corpuscles of one individual are introduced into the circulation of another individual of the same species. This was done by injecting an ox intravenously with from 2 to 4 litres of citrated blood taken from another individual, and subsequently carrying out daily examinations of the blood by means of exhausted sera. Altogether 8 cattle were injected and in all a similar course of events was

observed, the number of the foreign corpuscles in the circulation gradually diminishing until they disappeared—some 4 to 7 days after the injection. Shortly after the disappearance of the foreign corpuscles from the circulation, the blood serum began to acquire hæmolytic properties. These experiments emphasise in the most striking manner the definite individuality of the red blood corpuscles, as the injected corpuscles are not merely not accepted by their new host, but are regarded as definitely foreign, and in fact give rise to the formation of corresponding antibodies, in accordance with the general laws of immunity.

Todd and White concluded from these results that the red blood corpuscles of any individual (excluding the case of close blood relations) possess characters which differentiate them quite distinctly from the red blood corpuscles of any other individual, even of the same species ; and they suggested that this may be merely one example of a general law applying to the body cells in general, at any rate in the higher animals.

The writer was anxious to continue this work in England, but was faced with the difficulty that, apart from the large number of animals required for the production of sufficiently polyvalent sera, the usual small laboratory animals did not appear to lend themselves easily to the production of isolysins. By a happy chance, however, in the course of the preparation of an anti-fowl plague serum, it was found that the injection of fowls with fowl's corpuscles, although not giving rise to any obvious isolysin, leads to the formation of a very marked iso-agglutinin, and it was decided to make use of this iso-agglutinin for investigations on similar lines to those made with the isolysins.

Landsteiner and Miller (1924–5) had already made some experiments on the blood corpuscles of fowls, but used a hetero-agglutinating serum, prepared by immunising rabbits with washed fowl's corpuscles. By this means they were able to recognise eight different types of corpuscle, and stated that further experiments suggested a still greater multiplicity.

Experimental.

Preparation of the Iso-agglutinating Sera.

The iso-agglutinating sera used were prepared by the injection of fowls with the whole citrated blood of other fowls, and most of the birds immunised, as well as those used for providing the necessary blood, were Plymouth Rocks bred on the Institute's farm at Mill Hill. They were large healthy birds, although the stock was considerably in-bred.

The injections (10 to 20 c.c.) were usually made into the muscles of the breast, but in some cases intravenous or intraperitoneal injection was used. The route employed, however, did not seem materially to affect the production of the antibodies. The injections were repeated at intervals of from 7 to 10 days, and the birds were bled for serum about a week after the last injection.

The iso-agglutinins did not appear very rapidly in the blood of the immunised birds, and as a rule a minimum of five injections of blood was necessary in order to obtain a reasonably powerful serum. Commonly for the preparation of polyvalent sera a number of birds (6 to 12) were bled at the same time and their sera pooled, filtered, and kept in cold storage (0° C. approximately) until required for use. As the individual sera were highly iso-agglutinating it was, of course, essential to free them from any red blood cells, by sedimentation or the centrifuge, before mixing.

Titration of the Iso-agglutinating Sera.

While a hæmolytic serum can be titrated with some degree of accuracy, the determination of the strength of a hæmagglutinating serum is less easy, as it is difficult to establish a fixed and sharp end-point at which agglutination can be said to be complete. After numerous trials a macroscopic method was adopted as giving the most satisfactory results. The tests were made on glass plates, ordinary photographic half-plates being found very convenient for the purpose. The plates, after rubbing with cotton-wool moistened with ether to remove grease, were passed through a clean Bunsen flame, and their surfaces were then divided into squares of 25×25 mm. by means of a camel hair brush dipped in melted paraffin wax, twelve of these squares being conveniently made on such a plate.

The emulsion of red blood corpuscles was prepared by making a 5 per cent. dilution of citrated blood in normal saline, centrifuging, and replacing the supernatant fluid by saline. The presence of a small amount of citrate, however, appears to have no marked action on the process of agglutination, so that the washing of the corpuscles seems to be unnecessary and in some cases was dispensed with.

In making the tests two drops each of the corpuscle suspension and of the dilution of serum were placed together in one of the small squares and mixed by gently rocking the plate, or if necessary with platinum wire. Rocking was continued and the agglutination observed, if necessary with a hand lens, the final result being noted at the end of 15 minutes.

The process of agglutination under these conditions is interesting to watch ;

the mixture when freshly made, observed with a hand lens, shows merely a very fine and uniform granularity, but otherwise appears quite homogeneous. After a varying time, depending on the strength of the serum, the granularity becomes more evident and takes the form of a fine clumping, which increases until it is visible to the naked eye. This process proceeds until the cells are practically all agglutinated into large clumps floating in a clear fluid. Up to this time, if the plate has been steadily rocked, the clumps have shown no tendency to adhere to the glass, but move freely with the fluid ; then, rather suddenly, a change takes place ; the clumps begin to stick to the glass and in a very short time are firmly fixed there, so that rocking the plate merely rocks the clear fluid in which the clumps were previously suspended. The occurrence of this phenomenon forms a useful end-point, indicating complete agglutination. The tests were carried out on the bench at ordinary room temperature (20° C. approximately).

In the earlier tests in which this method was used, it was noticed that, when the area of a particular square was accidentally made greater than usual, the agglutination appeared to take place more rapidly in this square. In order to ascertain if there was a definite relation between the area of the square and the rapidity of agglutination, a couple of diverging lines were drawn on a plate with melted wax and connected by transverse lines, so as to form a series of squares of different sizes. Two drops of a suitable dilution of an iso-agglutinating serum were placed in each square and to each were then added two drops of a 5 per cent. suspension of corpuscles. The plate was steadily rocked and the time when agglutination was complete in each square noted. The result was as follows :—

Square	No. 1.	Area	234	sq. mm.	Agglutination	complete in	26	minutes.
,,	,, 2.	,,	336	,,	,,	,,	18	,,
,,	,, 3.	,,	497	,,	,,	,,	12	,,
,,	,, 4.	,,	665	,,	,,	,,	9	,,
,,	,, 5.	,,	920	,,	,,	,,	6	,,

It is thus evident that the rapidity of agglutination increases with the area over which the mixture is spread, so that in carrying out the tests it is important that squares of equal areas be used. No difficulty was experienced from the presence of normal iso-agglutinins, which are only exceptionally met with in fowls.

Influence of the Individuality of the Injected Corpuscles on the resulting Immune Iso-agglutinins.

In order to determine the result of immunising a fowl with the corpuscles of a single individual, two birds were set aside and one of these was immunised with the blood of the other. Five injections of approximately 20 c.c. of the whole blood were given at weekly intervals, the first injection being intraperitoneal and the remainder intramuscular. A week after the last injection the fowl was bled and the serum tested on the corpuscles of a number of fowls, as follows:—

Table I.—Agglutinating Action of the Serum of a Fowl (No. 147) which had been Immunised with the Corpuscles of another Fowl (No. 146).

Corpuscles of fowl No.	Dilutions of the serum.								
	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.
146	C.	C.	C.	C.	C.	C.	C.	++	—
R.28	+	—	—	—	—	—	—	—	—
C.1	C.	C.	+++	+++	—	—	—	—	—
C.2	C.	C.	+++	+	—	—	—	—	—
C.3	C.	C.	C.	+++	+	—	—	—	—
C.4	C.	C.	+++	++		—	—	—	—
C.5	C.	C.	+++	+++	—	—	—	—	—
C.7	C.	*a.c.*	+++	+++	—	—	—	—	—
C.8	C.	C.	+++	*a.c.*	—	—	—	—	—

C. = complete agglutination.
a.c. = almost complete agglutination.
+++ = very marked agglutination.
++ = marked agglutination.
+ = trace of agglutination.
— = complete absence of agglutination.

It will be seen that the immune serum is very much more highly agglutinating for the corpuscles used for its production than for those of other fowls, so that it is clear that the organism is able to recognise the injected cells as foreign to itself and elaborates corresponding antibodies.

Incidentally, in connection with this test, it was a matter of interest to know whether the exhaustion of the immune serum with the corpuscles used for its production would remove the agglutinating power, not only for these corpuscles, but also for the corpuscles of other fowls. This was found to be the case; the serum after exhaustion with these cells was quite without action on the corpuscles of a series of other individual fowls on which it was tested.

Three fowls were now immunised with the corpuscles of 12 others, and their

sera then separately tested on the cells of a number of fowls. In each of these three cases the serum was much less specific than the serum of the fowl immunised with the corpuscles of a single fowl only. This is, of course, to be expected, as the corpuscles of different individuals constitute different antigens (or groups of antigens), so that the immunisation of a fowl with the cells of a number of individuals naturally results in the formation of a greater variety of antibodies, and the resulting immune serum is more polyvalent.

Influence of the Individuality of the Animal into which the Corpuscles are injected on the resulting Immune Iso-agglutinins.

To compare the results obtained in different individuals immunised with the same corpuscles three fowls were immunised in parallel, *i.e.*, each fowl received the same dose of the same corpuscles on the same day, and after five injections, at intervals of about a week, the birds were bled for serum, each bird having received the blood of five fowls.

The immune sera of these three birds was then tested on the corpuscles of a number of other fowls.

Table II.—Comparison of the Agglutinating Action of the Sera of three Fowls all Immunised with the Blood of the same (five) individuals.

(In each case equal volumes of the undiluted serum and of a 5 per cent. suspension of the corpuscles were used.)

Serum of fowl.	Corpuscles of fowls Nos.									
	129.	130.	131.	132.	812.	853.	857.	887.	133.	134.
No. 64	+++	+	++	+	—	+++	*a.c.*	+	—	—
No. 65	+	C.	—	C.	++	C.	C.	+	C.	C.
No. 66	+	C.	—	—	—	*a.c.*	*a.c.*	—	+++	—

This result shows the very different way in which each of the individual birds has responded to the injection of the same corpuscles, and demonstrates in a striking manner the important part played by the individuality of the animal producing the antibodies.

Preparation of Polyvalent Iso-agglutinating Sera.

It is clear from the preceding experiments that the polyvalency of an iso-agglutinating serum increases with (*a*) the number of individuals whose corpuscles are used for the production of the serum, and (*b*) the number of individuals

immunised with these corpuscles; so that, for the production of a highly polyvalent serum, it is desirable that both these factors should be made as high as possible. Accordingly 12 large fowls were selected and were each injected intramuscularly at weekly intervals with the mixed blood of several fowls. Altogether each of the birds had six injections and received the blood from 22 other birds. One of the fowls under immunisation had to be discarded, owing to an accident, but the remaining 11 were bled 10 days after the last injection. These sera were pooled and the resulting mixture was tested on the corpuscles of a number of different fowls, so as to determine its relative activity for the cells of the respective birds.

Table III.—Agglutinating Action of a Polyvalent Serum (No. 55/12) on the Corpuscles of different Individuals.

Corpuscles of fowl.	Dilutions of the serum.			
	1/8.	1/16.	1/32.	1/64.
No. 135	C.	C.	+++	—
No. 136	C.	*a.c.*	++	—
No. 137	C.	C.	++	—
No. 138	C.	*a.c.*	+++	—
No. 64	C.	C.	+	—
No. 65	C.	C.	+	—
No. 66	C.	C.	+	—
No. 141	C.	C.	+	—
No. 142	C.	C.	+++	—
No. 143	C.	C.	+++	—

Table III shows that the serum prepared in this way is markedly polyvalent and agglutinates the corpuscles of most fowls at a comparatively uniform titre. This particular serum has since been tested (without determining its exact titre) on the cells of more than 100 birds, none of which was a contributor to the antigenic corpuscles, and in no case has it failed to give a rapid and complete agglutination, showing that, even by the use of only 11 individuals for the immunisation, a very considerable degree of polyvalency is reached. This could presumably be increased by immunising a larger number of birds.

Exhaustion of Polyvalent Iso-agglutinating Sera.

A highly polyvalent serum being thus obtained, it was now possible to utilise this for "exhaustion" experiments. In order to exhaust the serum for the corpuscles of a particular fowl a convenient volume was mixed with about a fifth of its volume of the washed red cells of the fowl in question, thoroughly

mixed by shaking, and allowed to stand for at least 15 minutes at room temperature, when it was centrifuged and the supernatant serum pipetted off. This process was repeated until the serum no longer showed any trace of agglutination when mixed with an equal volume of a 5 per cent. suspension of the corpuscles and rocked on a glass plate for 15 minutes. When this point was reached one further exhaustion was made, as a precaution, and the serum then stored at 0° C. until required for use. The number of exhaustions required varied with the strength of the serum and with the individual corpuscles, but in the case of the polyvalent serum referred to above, five exhaustions were usually found sufficient.

In order to test the effect of exhaustion, a polyvalent serum, prepared by immunising 11 fowls with the blood of 16 other individuals, was exhausted with the corpuscles of a single fowl (No. 102) and the exhausted serum tested on the corpuscles of 10 other birds (*vide* Table IV).

Table IV.

Agglutinating Action of Serum (No. 55/12) on the Corpuscles of Fowl No. 102 *before* Exhaustion with these Corpuscles.

Corpuscles of fowl.	Dilutions of serum.					
	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.
No. 102	C.	C.	C.	C.	++	—

Agglutinating Action of Serum (No. 55/12) *after* Exhaustion with the Corpuscles of Fowl No. 102.

Corpuscles of fowl.	Dilutions of serum.					
	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.
No. 135	C.	C.	C.	C.	+++	+
No. 136	C.	C.	C.	C.	+	—
No. 137	C.	C.	C.	C.	++	—
No. 138	C.	C.	C.	C.	+++	—
No. 133	C.	C.	C.	C.	*a.c.*	—
No. 134	C.	C.	C.	C.	*a.c.*	—
No. 812	C.	C.	*a.c.*	+++	—	—
No. 853	C.	C.	C.	C.	*a.c.*	+
No. 857	C.	C.	C.	C.	*a.c.*	+
No. 887	C.	C.	C.	*a.c.*	++	—
No. 102 (control)	—	—	—	—	—	—

The results of the test show that the exhausted serum, though absolutely without action on the cells for which it was exhausted, in most cases had lost comparatively little of its agglutinating power for the corpuscles of other individuals.

A large number of experiments made by exhausting samples of the same polyvalent serum with the corpuscles of different individuals gave similar results, although the corpuscles of some individuals were found to remove more of the agglutinins than others.

The Recognition of the Individual by Means of Exhausted Sera.

In order to determine whether such exhausted sera could be used for the recognition of a particular individual, as was found by Todd and White (1910[1]) to be possible in cattle by the use of exhausted isohæmolytic sera, a polyvalent iso-agglutinating serum was exhausted with the corpuscles of a particular fowl and tested on the corpuscles of as many different fowls as possible. Up to the present the cells of 89 other birds have been examined, and in all cases have been completely agglutinated by the exhausted serum. In most cases complete agglutination was produced by a 1 in 8 dilution, and in only two instances was it necessary to use a dilution of the serum as strong as 1 in 2. As the stock of fowls at the Institute is considerably inbred it is not improbable that these two birds were in some degree related to the fowl whose corpuscles were used for the exhaustion of the serum. The influence of close blood relationship is considered later.

While these numbers are not sufficiently large to warrant a general conclusion, it is probably safe to assume that a highly polyvalent iso-agglutinating serum, exhausted with the corpuscles of a particular fowl, is still capable of agglutinating the corpuscles of any other individual fowl (not a close blood-relation) and so behaves as a specific reagent for the individual in question.

It must be borne in mind that the polyvalency of the serum used in these experiments was still comparatively low, and that it could presumably be considerably raised by the use of a larger number of individuals for its production.

Exhaustion of Polyvalent Iso-agglutinating Sera with the Corpuscles of more than one Individual.

If a polyvalent serum which has been exhausted with the corpuscles of one individual is further exhausted with the corpuscles of a second, its agglutinating power in general is naturally much diminished and, for the corpuscles of certain

individuals, may have entirely disappeared. As a rule, however, it still agglutinates the cells of most individuals, and in a series of 10 tests made with polyvalent sera exhausted with the corpuscles of two fowls and then tested on the cells of other birds, in only 11 cases out of 78 (*i.e.*, 14 per cent.) was there a complete lack of agglutination.

Even after exhaustion with the red cells of three individuals the serum generally still shows a considerable degree of activity for certain corpuscles. In one experiment a polyvalent serum was successively exhausted with the corpuscles of three fowls. A similar volume of the same serum was then exhausted in the same way with the corpuscles of the same individuals, but in this case the order in which the exhaustions were made was reversed. After the first and second exhaustions the two sera—tested on a number of different corpuscles—naturally showed very marked differences, but after the third exhaustion (*i.e.*, when each had been exhausted by the same corpuscles, though in a different order) the two sera were indistinguishable. Further experiments confirmed this, and showed that the final result of exhausting a serum with the corpuscles of two or three individuals is not affected by the order in which the exhaustions are carried out.

Heredity Experiments.

It was obviously very desirable to examine and compare the behaviour of the red cells of closely related fowls by means of these iso-agglutinating sera, as was done by v. Dungern and Hirschfeld (1910) with iso-agglutinins in dogs, and by Todd and White with isolysins in cattle.

Attempts to obtain families of chicks of known parentage were unsuccessful, so that it was necessary to breed them specially for the purpose, and the writer is much indebted to Major G. W. Dunkin, M.R.C.V.S., who very kindly arranged for this to be carried out at the Institute's farm at Mill Hill and directed the work. Plymouth Rock birds were used, and three couples, each consisting of a cock and hen, were isolated in separate runs. The eggs from each pair were collected, marked, and incubated. On hatching each chick was marked with a coloured ring (red, white or blue, according to the family). The chicks were brought up by foster-mothers and were first bled from the vein when about a month old. They were then further marked with numbered rings, so that the individual chicks could be identified.

Up to the present 61 chicks have been reared, 19 of which belong to the red, 22 to the white, and 20 to the blue family. The corpuscles of each of these

chicks were tested for agglutination with the same polyvalent serum, which had been exhausted in different ways, viz. :—

(*a*) Serum exhausted with the corpuscles of the father.

(*b*) Serum exhausted with the corpuscles of the mother.

(*c*) Serum exhausted with the corpuscles of *both* the father and mother.

The tests were made in the usual way, two drops of a 5 per cent. suspension of the corpuscles being mixed with two drops of the undiluted exhausted serum.

Examining the results of these tests (Tables V, VI and VII), and considering firstly the action of the sera exhausted *separately* for the cells of the father and for those of the mother, we see that in the majority of cases the red cells of the chicks show a distinct individuality and can be distinguished from those of either parent by suitably exhausted sera.

In a number of cases, however, particularly in the "white" and the "blue" families, the corpuscles of the chick are indistinguishable from those of one parent, though quite different to those of the other. Thus in the white family (Table VI) the corpuscles of 8 of the 22 chicks are no longer agglutinated by the serum after it has been exhausted with the corpuscles of the father, while they are still agglutinated by the serum which has been exhausted by the mother's corpuscles. In the same way the cells of 7 of the 20 chicks of the blue family are serologically indistinguishable from those of their mother, although definitely different to those of their father. In only two cases did both the serum exhausted for the father and the serum exhausted for the mother fail to agglutinate the cells of the chick.

The outstanding fact, however, shown by these tests is the complete lack of agglutinating action of the serum which has been exhausted for the cells of *both* the father and mother; with one single exception, this serum showed not the faintest trace of agglutination with the corpuscles of any of the 61 offspring. The case of the single exception was particularly interesting. This chick (No. 9) was supposed to belong to the blue family, but the fact that its corpuscles were rapidly and completely agglutinated by serum exhausted for the cells of both its reputed parents, at once raised serious doubts as to the accuracy of its pedigree, and its corpuscles were therefore examined with respect to their relation with those of the other two families.

The details of the test are given here (Table VIII) and are interesting as showing how the use of exhausted sera may give valuable assistance in deciding the question of doubtful parentage.

Table V.—Examination of the Corpuscles of the Chicks of one Family with a Serum Exhausted for the Corpuscles of their Parents. Red Family.

Corpuscles of chick.	Serum exhausted for corpuscles of father.	Serum exhausted for corpuscles of mother.	Serum exhausted for corpuscles of father and mother.
No. 1	C.	C.	—
No. 2	C.	+++	—
No. 3	—	C.	—
No. 4	C.	C.	—
No. 5	+++	—	—
No. 6	C.	—	—
No. 7	C.	C.	—
No. 8	C.	+	—
No. 28	+++	+	—
No. 29	C.	C.	—
No. 30	C.	C.	—
No. 31	C.	—	—
No. 32	—	+	—
No. 33	C.	C.	—
No. 47	C.	C.	—
No. 48	C.	C.	—
No. 49	*a.c.*	*a.c.*	—
No. 50	C.	C.	—
No. 51	C.	*a.c.*	—

Table VI.—Examination of the Chicks of one Family with a Serum exhausted for the Corpuscles of their Parents. White Family.

Corpuscles of chick.	Serum exhausted for corpuscles of father.	Serum exhausted for corpuscles of mother.	Serum exhausted for corpuscles of father and mother.
No. 19	—	—	—
No. 20	C.	C.	—
No. 21	—	C.	—
No. 22	C.	C.	—
No. 23	—	C.	—
No. 24	*a.c.*	C.	—
No. 25	—	—	—
No. 26	—	C.	—
No. 27	C.	C.	—
No. 34	*a.c.*	C.	—
No. 35	*a.c.*	C.	—
No. 36	C.	C.	—
No. 37	—	C.	—
No. 38	C.	C.	—
No. 39	+++	++	—
No. 40	—	C.	—
No. 41	+++	++	—
No. 52	C.	C.	—
No. 53	—	C.	—
No. 54	C.	C.	—
No. 55	—	C.	—
No. 56	—	C.	—

Table VII.—Examination of the Chicks of one Family with a Serum exhausted for the Corpuscles of their Parents. Blue Family.

Corpuscles of chick.	Serum exhausted for corpuscles of father.	Serum exhausted for corpuscles of mother.	Serum exhausted for corpuscles of father and mother.
No. 9	C.	C.	C.
No. 10	+++	—	—
No. 11	+++	—	—
No. 12	+++	—	—
No. 13	*a.c.*	C.	—
No. 14	*a.c.*	—	—
No. 15	C.	C.	—
No. 16	C.	—	—
No. 17	*a.c.*	C.	—
No. 18	C.	—	—
No. 42	C.	C.	—
No. 43	C.	C.	—
No. 44	C.	C.	—
No. 45	C.	C.	—
No. 46	C.	—	—
No. 57	C.	C.	—
No. 58	C.	++	—
No. 59	C.	C.	—
No. 60	C.	+	—
No. 61	C.	++	—

Table VIII.—Examination of the Corpuscles of Chick No. 9 with Sera exhausted for the Parents of the three Families.

A 5 per cent. suspension of the red cells of this chick was tested with three dilutions of each of the sera. Tests made on a glass plate in the usual way.

With Serum exhausted for the Parents of the Red Family.

Serum.	Dilutions of serum.		
	1/1.	1/2.	1/4.
Serum exhausted for father	C.	C.	*a.c.*
,, ,, mother	C.	C.	C.
,, ,, father and mother	C.	C.	+++

Table VIII.—(continued.)

With Serum exhausted for the Parents of the White Family.

Serum.	Dilutions of serum.		
	1/1.	1/2.	1/4.
Serum exhausted for father	++	+	—
,, ,, mother	C.	*a.c.*	—
,, ,, father and mother	—	—	—

With Serum exhausted for the Parents of the Blue Family.

Serum.	Dilutions of serum.		
	1/1.	1/2.	1/4.
Serum exhausted for father	C.	C.	C.
,, ,, mother	C.	C.	C.
,, ,, father and mother	C.	C.	*a.c.*

It is at once evident from these results that the chick shows no relation to the parents of either the red or the blue families, but can, with a high degree of probability, be attributed to the white family, and this for two reasons: (1) Its corpuscles are entirely unaffected by the serum exhausted for the father and mother of this family, as in the case of the remaining 60 chicks, and (2) its corpuscles show only a slight agglutination with the serum exhausted for the father of this family, instead of the rapid and complete reaction always observed with the cells of a non-related individual. It would therefore appear highly probable that this chick really belongs to the white family, and not to the blue, and that a mistake had been made in labelling the egg.

Discussion.

It should be noted that, while Todd and White's work was carried out on non-nucleated mammalian corpuscles, by the use of hæmolytic methods, the experiments described above deal with nucleated avian corpuscles, which were studied by means of agglutination.

In spite of these differences the results are in complete agreement, and show that, where close blood relationship is not involved, in both cases the red blood cells of any one individual possess characteristics differentiating them quite

definitely from the red blood cells of any other individual of the same species, and, moreover, that this individuality can be clearly demonstrated by comparatively simple serological methods.

When we realise that it is possible to distinguish the corpuscles of a particular fowl from those of any other existing non-related fowl, as appears to be the case, we see the almost infinite range of specificity involved. That the appreciation of these exquisitely fine differences is, however, not beyond the power of the organism is seen when a normal fowl is injected with the red cells of another fowl. The injected cells are apparently recognised by their new host as foreign to its organism and are appropriately dealt with, the necessary mechanism for their elimination being forthcoming. Still, it is none the less remarkable that such astonishingly small differences as must exist between the red cells of two individuals of the same species can be recognised by means of artificially prepared immune sera, and the results obtained show the peculiar value of iso-antibodies for the investigation of the finer details of specificity existing within the species.

Being in complete ignorance of the nature of these finer details of specificity, we are naturally in the dark as to how the iso-antibodies act; but a consideration of the way in which they arise is perhaps instructive. We may consider in the first place the case of a fowl injected with blood from an animal of a different species—for instance a rabbit. One of the outstanding points in the behaviour of an animal is the jealousy with which it defends the constitution of its own protoplasm. The activities of the defensive mechanisms of the fowl are here primarily concentrated on the denaturation and removal of the definitely foreign rabbit protein, and a series of antibodies is elaborated for this purpose. These must naturally be specific for the various proteins of the injected blood, and also "species specific" for the rabbit.

If, on the other hand, a fowl is injected with fowl's blood, the danger from the presence of foreign proteins is absent and the organism is, so to speak, free to devote its energies to the problem of dealing with the injected cells which, though not definitely foreign, can still be recognised as different to those of their new host. The fact that the injected cells are not definitely foreign probably renders their removal less easy, as it cannot be effected by means of comparatively simple "species specific" antibodies (*e.g.*, precipitins), and some more complex, and possibly quite different, form of mechanism is presumably required. That this is the case would seem to be indicated by the greater difficulty generally experienced in preparing iso-antisera as compared with hetero-antisera, although this varies within somewhat wide limits in

different species. Thus in the ox, after a single injection of ox corpuscles, the injected cells have completely disappeared in from 4 to 7 days, and shortly after their disappearance the presence of iso-hæmolysins can be observed in the blood. In the fowl, on the other hand, several injections of blood appear to be necessary, and in a number of other species it has not yet been possible to demonstrate the presence of iso-antibodies, even after repeated injections of large volumes of blood continued over some time.

Whatever may be the mechanism by which they act, the iso-antisera show themselves extraordinarily selective, so that a particular serum when tested on the red cells of a number of individuals may manifest a different degree of activity for the corpuscles of practically each individual. Another characteristic, which was already noticed by Ehrlich and Morgenroth in their early work, is that no two individual sera are entirely alike in their relative selectivity.

A further point, which appears quite clearly from the examination of the red cells of a large number of fowls with different iso-agglutinating sera, is that the corpuscles of different individuals show marked differences in their behaviour to the sera. In short, it appears that no two iso-agglutinating sera are identical in their action on the corpuscles of different individuals; nor do the red cells of any two individuals behave in exactly the same way towards the same serum.

This is entirely in accordance with the ingenious hypothesis put forward by Ehrlich, that every red blood-cell possesses a large number of haptophore groups, regarded as analogous to the side-chains of a complex organic molecule, each of which is able to combine in the animal body with fitting receptors. While the side-chain theory in its entirety may perhaps be regarded more as a schematic presentment of the collective phenomena of immunity than as a representation of the actual conditions, and must not be taken too literally, it is difficult to see how the serological behaviour of the red blood cells is to be explained without postulating a large number of separate affinities, and the results obtained in the preceding experiments may be conveniently stated in the conventional terms of this theory.

Using Ehrlich's nomenclature, then, we may say that the red cell, when examined by serological methods, can be shown to possess a large number of different receptors, each of which is antigenic, so that the corpuscle must be regarded as a multiple antigen. The receptors are different in the red cells of every individual—either in character, number, or arrangement—although many receptors are common to many individuals. As each receptor gives rise to a corresponding antibody, and as the amount of such antibody formed

presumably depends, to some extent at least, on the number of the corresponding receptors, it follows that immunisation with the red cells of different individuals will produce different antisera. Similarly the exhaustion of the same polyvalent antiserum with the corpuscles of different individuals will leave different sets of antibodies in the different exhausted sera. It is also evident that if an animal is immunised with the cells of a single individual, exhaustion of the immune serum with the cells of this same individual must necessarily remove *all* the antibodies formed in the serum, while exhaustion with the corpuscles of any other individual will *not* do so. Moreover, in the case of a polyvalent immune serum we see why exhaustion with the red cells of one, two, or even three individuals need not necessarily remove all the antibodies from the serum.

Again, in two different individuals acting as sources of antibodies, the side-chains of the antibody-producing tissues may be expected to differ in kind, or in relative abundance; so that injection into each individual of the same corpuscles would presumably provoke qualitatively or quantitatively different immune reactions to the corpuscular receptors, with resulting differences in the reactions of the two iso-agglutinating sera produced.

In the course of their work on the isolysins of the goat, Ehrlich and Morgenroth noticed that two goats injected with similar amounts of the same blood developed *different* isolysins, which showed that the constitution of the isolysin is dependent on the individuality of the animal in which it is formed. This observation does not appear to have attracted the attention which it deserved. It is a matter of common experience that different individuals of the same species immunised with similar doses of the same foreign antigen (*e.g.*, bacteria, toxins, etc.) often yield anti-sera of very different strengths, as the degree of response varies within somewhat wide limits in different individuals. A similar occurrence is observed in the case of iso-antisera, and, in one experiment where two fowls were immunised in parallel with corpuscles of the same bird, the iso-agglutinating power of the serum in one was found to be approximately 30 times that in the other, when tested on the corpuscles with which both birds had been immunised. These are, however, merely quantitative differences and are to be distinguished from the very striking qualitative differences referred to.

The fact that it is possible in certain animals to distinguish the red cells of any individual from those of any other (not closely related) individual naturally suggests the question whether the same can be done in the case of man, where such recognition of the individual would be of immediate practical value.

Unfortunately, apart from the practical objections to the preparation of iso-antisera in man, human corpuscles when transfused appear to persist in the circulation for somewhat long periods (Ashby, 1919) without giving rise to demonstrable antibodies, although the accidents which have been recorded by several workers as following repeated transfusions with the same blood, suggest that this behaviour is not invariable. In man the problem is also complicated by the fact that human red cells, when examined by means of the iso-agglutinins normally occurring in the blood, are found to arrange themselves into four great groups. The characters of these groups are racial and are transmitted according to Mendelian laws, but we are at present quite in the dark as to their significance. These group differences are, however, on an entirely different plane from the immeasurably finer individual differences which must undoubtedly exist, but would probably require the artificial production of immune iso-agglutinins or isolysins for their detection. The subdivision of group A and Landsteiner and Levene's recent work (1928), showing the presence of a number of more or less definite agglutinable factors within the four groups, all point in this direction.

The heredity experiments here described are still in an early stage, as the breeding of an adequate number of chicks naturally takes some time, and the chicks so far obtained are not yet large enough to yield an adequate amount of blood for exhaustion experiments, so that it has not been possible to examine the behaviour of the corpuscles of the parents with sera exhausted for the cells of the chicks. The results already obtained are, however, of interest. It has been shown that, after exhaustion with the red cells of any one fowl, the exhausted serum invariably agglutinates the corpuscles of all other (unrelated) individuals. It therefore follows that a negative result with the corpuscles of any particular fowl is evidence of close blood relationship between the fowl in question and the fowl whose red cells were used for the exhaustion of the serum.

Examination of the results of the agglutination reactions recorded in Tables VI, VII and VIII (excluding chick No. 9, whose parentage is doubtful) shows that, of the total of 60 chicks, the corpuscles of :—

10 (*i.e.*, 17 per cent.) were negative only to the serum exhausted for the father.

10 (*i.e.*, 17 per cent.) were negative only to the serum exhausted for the mother.

2 (*i.e.*, 3 per cent.) were negative both to the serum exhausted for the father and also to the serum exhausted for the mother.

38 (*i.e.*, 63 per cent.) were negative neither to the serum exhausted for the father nor to the serum exhausted for the mother.

60 (*i.e.*, 100 per cent.) were negative to the serum exhausted *both* for the father and the mother.

Considering in the first place the case of the serum exhausted for *one* parent only, we see that it is possible to establish the relationship of the chick to the father in 17 per cent., and to the mother in another 17 per cent., of the chicks ; that is to say the corpuscles of 34 per cent. of the chicks show a definite resemblance to one or other parent. In the remaining 66 per cent. the corpuscles do not show any such exclusive resemblance to those of either parent. On the other hand, when the serum is exhausted for the corpuscles of *both* parents, it is entirely without action on the corpuscles of the chicks in all cases.

This behaviour of the cells of different chicks appears at first sight somewhat confusing, but it can be very simply represented in terms of the side-chain theory. For this purpose it is only necessary to postulate the existence of three different groups of affinities (A, B and C), of which A is peculiar to the father, B to the mother, and C common to both. The polyvalent serum contains of course antibodies to A, B and C, so that if it is exhausted with the corpuscles of the father the antibody for B only will remain. Similarly exhaustion with the cells of the mother will leave only the antibody for the receptor A. If, however, the serum is exhausted with both corpuscles all the antibodies (*i.e.*, the antibodies for A, B and C) will be removed.

On this hypothesis the behaviour of the different corpuscles to the exhausted sera will be as shown below :—

Table VIII.—Showing how the behaviour of the corpuscles of the chicks may be represented as determined by the receptors which they possess, assuming that the corpuscles of the father have receptors A and C, and the corpuscles of the mother the receptors B and C, then the corpuscles of the chicks will behave according to the receptors which they themselves possess, as shown below :—

Receptors possessed by corpuscles of chick.	Agglutination shown with—		
	Serum exhausted with corpuscles of father.	Serum exhausted with corpuscles of mother.	Serum exhausted with corpuscles of *both* father and mother.
A only (or A and C)	−	+	−
B only (or B and C)	+	−	−
C only	−	−	−
A and B	+	+	−

In reality the conditions are very much less simple, as the red cells undoubtedy possess a multiplicity of affinities or receptors, but it is interesting to note that the hereditary transmission of the characters of the red cell is capable of a comparatively simple representation.

A very striking point in these heredity experiments is the fact that a serum, exhausted for the cells of *both* the father and mother, is invariably without action on the cells of the chick, showing that the corpuscles of the chick have no receptors not possessed by at least one of their parents. If this observation is confirmed by further experiment it would seem to show that there is no appearance, in the corpuscles of the chick, of antigenic characters which have been transmitted through the parents in a latent form.

By the use of a serum exhausted for the cells of *both* the father and mother we are enabled to go a good deal further towards establishing the parentage of any particular chick ; since, if the red cells of the chick are agglutinated by the serum exhausted for the corpuscles of *both* the reputed parents, it can be confidently assumed that the attribution of parentage is incorrect as regards one or both parents. Unfortunately if the cells of the chick are not agglutinated by the serum it is impossible to draw any definite conclusion, as the exhaustion of a serum with the corpuscles of two individuals may, in some cases, weaken it to such an extent as to render it inactive even for the cells of certain unrelated individuals. It seems not improbable, however, that this difficulty will be overcome by the use of more powerful and more polyvalent sera.

It is not easy to form a conception of this remarkable specificity of the cells of the individual as limited to the red blood corpuscles, and one is naturally led to enquire whether the individuality of the red cell is not simply one example of a general rule applying to all the cells of the body. Unfortunately the fixed cells do not lend themselves to serological investigation with the same ease as the red blood corpuscles, but a considerable amount of evidence is available in other directions, particularly from experiments on the transplantation of skin and other tissues. As the result of this work, which has been well summarised by Schöne (1912) and Neuhof (1923), it is now generally acknowledged that, in the mammalia, while auto-transplantation is commonly successful and heterotransplantation under ordinary conditions invariably unsuccessful, homoio-transplantation rarely succeeds, except in special circumstances such as in certain cases of close blood-relations.

In considering the lack of success usually attending homoio-transplantation in the higher animals it is impossible to resist the conviction that, generally speaking, the cells of the body of any one individual, as in the case of the red

blood corpuscles, are specifically different from the similar cells of any other non-related individual of the same species ; this individual specificity being so marked that the tissues of any two individuals are definitely incompatible, except where close blood-relationship is involved.

According to this view, which has been strongly advocated by L. Loeb, the results obtained in transplantation experiments are capable of logical explanation in accordance with the general laws of immunity. For example, in Schöne's experiments with rats and mice, homoio-transplantation in unrelated animals was never successful. The grafts might appear viable and fresh even as long as 14 days after transplantation, but then a rapid degeneration would invariably set in resulting in the desiccation and discharge of the transplant.

There can be little doubt that the sudden change in the behaviour of the grafts after this period coincides with the appearance of antibodies formed by the host in response to the presence of the foreign tissue. This view is supported by the fact that immunity to certain transplantable tumours may be induced by previous treatment of the animals with normal tissue of their own species and also by Fleisher's (1918) experiments on the different results obtained by transplanting guinea pig's kidney into normal guinea pigs and into guinea pigs previously immunised with guinea pig's kidney.

As regards the influence of blood-relationship on the results of homoio-transplantation, Thiersch, in his first systematic study of this problem in man, noted that skin transplants did not succeed in individuals who were not blood-relations, and his work has been confirmed in experiments on animals by a large number of workers—notably by Schöne and L. Loeb. If we assume that the tissue cells of closely-related individuals may resemble one another in possessing certain common haptophore groupings, as happens in the case of the red blood corpuscles of such relations, the observed results of transplantation are capable of reasonable explanation.

The application of the methods of immunity to the subject of biological specificity by a number of workers, and particularly by Landsteiner and his colleagues, and by Dochez, Avery, Heidelberger, and others, in recent years has shown this specificity to be chiefly, if not entirely, dependent on the chemical structure of the protein or protein-hapten complexes, so that chemical-species and organ-specificities must all be regarded as essentially chemical in character.

If the individual specificity of the cells is to be attributed to differences in the chemical constitution of their protein, or protein-hapten complexes, this must be capable of an almost incalculable variation. This is theoretically

quite possible, and perhaps not improbable, but even so it is difficult to understand why the cells of one individual should be so easily distinguished from those of another individual of the same species by comparatively simple methods, while the proteins of certain different species, such as the albumins of hen's and duck's eggs, which are known to be chemically different, can only be differentiated by the most refined methods of immunity (Dakin and Dale, 1919). Is this due to the complex nature of the cell, which renders it a highly multiple antigen, or is it rather to be attributed to a peculiar specificity of the iso-antibodies for individual differences within the species? These are matters which must remain for further investigation.

Conclusions.

1. It is possible in the domestic fowl, by means of simple immunity reactions, to differentiate the red blood corpuscles of any particular fowl from those of any other individual of the same species, except in certain cases where there is close blood-relationship; thus showing that the red cells of any individual possess specific characteristics distinguishing them from those of any other individual not a close blood-relation.

2. It appears probable, in the case of the higher animals, that this individual specificity of the red blood corpuscle is only one example of a general rule applying to most of the other cells of the body, and this view is supported by a considerable amount of evidence, particularly from the results of experiments on the transplantation of tissues.

3. A comparison of the red cells of chicks with those of their parents allows in some cases the actual identification of at least one of the parents, and in a large proportion of the chicks affords definite evidence of relationship. The experiments are, however, as yet in too early a stage to permit any definite estimate of the extent to which these methods are capable of application.

I desire to record my indebtedness to the Medical Research Council for the opportunity afforded me of working on this subject at the National Institute for Medical Research, and also to Capt. S. R. Douglas, F.R.S., in whose department of that Institute the work has been carried out.

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The Delayed Lethal Effect of Radium on Tissue Cultures in vitro.

By F. G. Spear, M.B.

(Communicated by Sir Frederick Andrewes, F.R.S.—Received November 11, 1929.)

(From the Strangeways Research Laboratory, Cambridge.)

Introduction.

The following experiments were made in order to study the delayed lethal effect of gamma rays on tissue cultures *in vitro*, using two different quantities of radium sulphate as the source of irradiation. The work forms part of a more extensive investigation into the causes of the destruction of certain types of new growth by means of radiotherapy. The expenses in connection with this study were met by a grant from the Medical Research Council, and my thanks are also due to the Radium Belge and Messrs. Watson & Sons for the loan of the radium employed.

Many workers have studied the effect of radium rays on unicellular organisms, and extensive work has been done on the irradiation of tissues *in vivo*. With the development of the technique of tissue culture *in vitro* a new and almost ideal experimental field was provided for the investigation of the action of radium on the cell.

Qualitative observations on the effect of radium on mitosis *in vitro* were begun by Canti and Donaldson (1923). Quantitative observations were made by Canti and Spear (1) (1927) and (2) (1929), who showed that after a

short exposure* to gamma irradiation, a marked fall occurred in the number of cells in mitosis (40 per cent. that of controls), and that this was followed by an increase (160 per cent. that of controls) which compensated almost exactly for the diminution seen during the first period subsequent to irradiation.

The present investigation concerns exposures to gamma rays of radium, which caused a delayed lethal effect on tissue cultures *in vitro*.

Technique.

The tissue was obtained from the choroid and sclerotic of chick embryos of 7 to 8 days' incubation. The cultures were grown in fowl plasma and chick embryo extract, on a No. 1 coverslip inverted over a hollow-ground slide, sealed with paraffin wax and incubated at 37° C. Growths of the first or second subculture were employed.

Exposures to irradiation were made in a special apparatus, called a radium lantern and described in a previous paper (Canti and Spear, 1927). It consists essentially of two discs of commercial lead, each capable of carrying one hollow-ground slide. The radium is arranged half-way between the two lead discs so that two cultures can be exposed at one time.

One series of experiments was made using 100 mg. radium as the source of irradiation; another series was made using 300 mg. radium. Throughout the experiments the radium acted at a constant distance of 0·5 cm. Specimens were brought from the culturing laboratory and placed in the lantern, which, with the radium, was maintained at a temperature of 37° C. Care was taken to prevent chilling during transference to and from the radium. For each culture exposed, a control culture was set aside for observation. Immediately after irradiation the specimens and their controls were subcultivated and returned to the incubator. Subcultures were then made every 48 hours, as long as the irradiated explants survived. In this subcultivation, the explant was not divided, but only "trimmed" and transplanted to fresh medium. The controls were similarly treated.

General Appearance of Cultures.

Control Cultures.—A few hours after cultivation, cells begin to wander out from the tissue into the surrounding medium by amœboid movement, until the fragment becomes surrounded by a more or less dense zone of cells. This zone consists of evenly distributed feather-like cells, which arrange themselves in columns radiating from the explant, like petals of a daisy. Cell division

* 2½ minutes' exposure to 100 mg. at 0·5 cm. distance using 0·5 mm. platinum filter.

is frequent in this zone after 12 hours' growth and the number of mitotic figures increases fairly rapidly up to about the 18th hour, thereafter remaining fairly constant in a given culture until the 40th hour of incubation.

Irradiated Cultures.—When normal explants of 24 hours' growth after the second subculture are exposed to 300 mg. radium at 0·5 cm. distance for 6 hours, they show an inhibition of mitosis, but no marked effect on cells in the vegetative condition. If subcultivated immediately after irradiation, cells begin to wander out from the explant into the medium within a few hours of incubation, and in less than 24 hours cells in division may be seen. Mitotic figures, however, are not nearly so numerous as in the corresponding controls, although emigration of cells is usually plentiful.

After one or two more subcultures mitosis is no longer seen, and while cells continue to wander out from the explant into the medium, the character of this zone of growth becomes conspicuously altered. Instead of elongated cells growing out from the fragment in evenly arranged columns, the cells are irregularly disposed about the medium in straggling lines, like withering grass. Many cells, especially at the outer edge of the zone of growth, lie quite isolated. Growth is scanty and individual cells of unusually large size are sometimes encountered; many cells are rounded and degenerate, whilst others are breaking down. With each subculture the explant shrinks, becoming slightly smaller. After four subcultures hardly any amœboid cells are seen around the tissue fragment and after the sixth subculture all emigration ceases.

In cultures which have been irradiated for periods of 12 hours, mitosis may be absent, although amœboid cells continue to emigrate even after two or three subcultures. In such preparations, giant cells are frequent, and degenerate and breaking-down cells are often seen.

It should be noted that a culture which exhibits no emigration of cells after 24 or 48 hours' growth following subcultivation, may, nevertheless, show a few migrating cells if it is kept untouched in the incubator for 3 or 4 days. In the present experiments a culture was considered "dead" when, after 48 hours' incubation following the last subculture, it showed either no emigration or not more than three cells projecting from the explant.

The cultures which survived irradiation were not carried beyond the 20th subculture (*i.e.*, 40 days' growth). The control cultures which were used merely to check the cultural conditions of the experiment, were not maintained after the death of the corresponding irradiated specimens.

Results of Experiments.

First Series of Experiments.—Cultures exposed in lantern to 100 mg. radium (filtered through 0·5 mm. platinum) at 0·5 cm. distance.

9 Hours' Exposure.—Eight specimens average 10 subcultures before death. Recovery occurred in one specimen which was subcultivated 20 times. It then showed almost complete recovery and compared favourably with its control. Subcultivation was then discontinued.

12 Hours' Exposure.—Six explants were carried to an average of nine subcultures. One specimen survived and was subcultivated 20 times.

18 Hours' Exposure.—Explants usually died at the ninth subculture. Mitosis was seen until the third subculture; one culture survived just over 3 weeks before death.

24 Hours' Exposure.—Migration of cells occurred on the average over a period of four subcultures. None occurred after the fifth. Hardly any mitosis was seen.

Second Series of Experiments.—Cultures exposed in lantern to 300 mg. radium (filtered through 0·5 mm. platinum) at a distance of 0·5 cm.

3 Hours' Exposure.—The majority of specimens were changed 10 times. Two specimens seemed about to die on the 19th day after irradiation but just survived the 10th subculture and thereafter showed gradual recovery. The explant enlarged in size, and by degrees the zone of growth lost its "scraggy" character and became normal in appearance. By the 20th subculture these specimens were similar in character to the corresponding controls.

6 Hours' Exposure.—Specimens as a rule died after six subcultures. Mitosis was seen in the zone of growth after the first subculture but not subsequently.

9 Hours' Exposure.—Four specimens reached an average of four subcultures. Two specimens were changed eight times.

12 Hours' Exposure.—Specimens survived, on the average, three subcultures before death. No mitosis was seen.

18 Hours' Exposure.—The majority of specimens died at the second subculture. One specimen was changed three times. No mitosis was seen.

24 Hours' Exposure.—Only one explant out of seven survived the first subculture.

These results are summarised in Tables I and II, and diagrammatically expressed in Charts I and II. In the charts, for the sake of uniformity, only six specimens are included for each exposure.

Discussion.

These results indicate that, while there is considerable variation in the resistance of individual cultures to the lethal action of gamma radiation, yet in general it may be said that the greater the intensity of irradiation the sooner is the lethal effect observed.

In the experiments with 300 mg. radium, the probable life of a culture is approximately doubled when the duration of exposure is halved. With 100 mg. radium halving the length of exposure has a less marked tendency to double the probable life of the culture. In this series the resistance of individual

Table I.—100 mgm. of radium filtered through 0·5 mm. platinum, acting at 0·5 cm. distance.

Hours of exposure.	Number of subcultures before death.									Average.
9	20*	15	14	11	11	10	10	9	9	11+
12	20*	11	10	10	9	8	7	—	—	9+
18	13	9	9	8	8	8	—	—	—	9
24	7	6	5	5	5	5	4	—	—	5
	Each figure has reference to one irradiated specimen.									

* Recovery of culture.

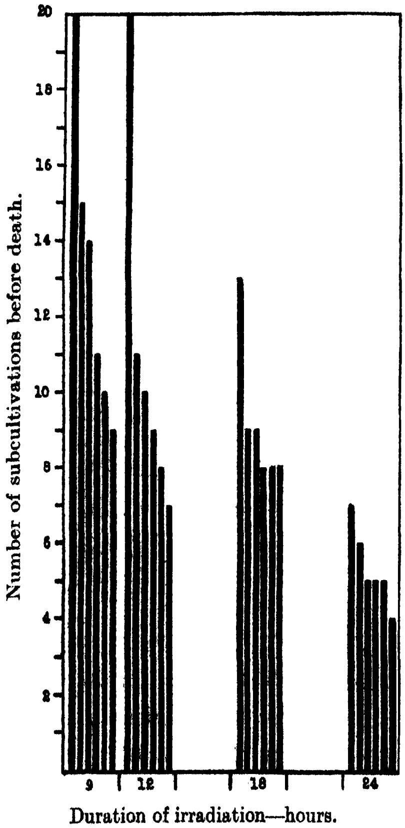

CHART I.—Figures of Table I arranged as a chart. 100 mg. radium acting at 0·5 cm. Each vertical represents life-history of one culture.

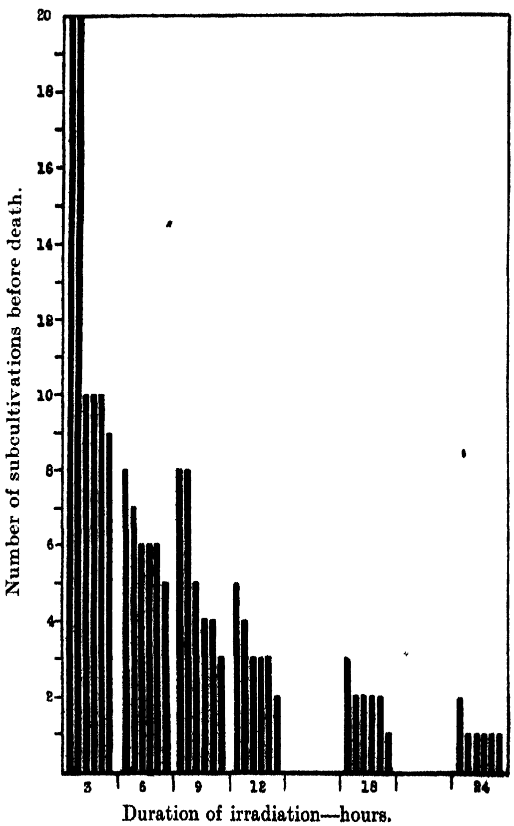

CHART II.—Figures of Table II arranged as chart. 300 mg. radium at 0·5 cm. Each vertical represents life-history of one culture.

Table II.—300 mgm. of radium filtered through 0·5 mm. platinum, acting at 0·5 cm. distance.

Hours of exposure.	Number of subcultures before death.								Average.
3	20*	20*	10	10	10	9	—	—	10+
6	8	7	6	6	6	5	—	—	6
9	8	8	5	4	4	3	—	—	5
12	5	4	3	3	3	3	3	2	3
18	3	2	2	2	2	2	1	—	2
24	2	1	1	1	1	1	1	—	1
	Each figure has reference to one irradiated specimen.								

* Recovery of culture.

cultures to the lethal action of radiation is more variable than that seen with the larger quantity of radium.

On comparing the action of the two quantities of radium, it is seen that an exposure to 300 mg. radium for 6 hours (1800 milligram-hours) produces a lethal effect on the average 6 days (three subcultures) earlier than in the case of an exposure to 100 mg. for a period of 18 hours (1800 milligram-hours).

It is interesting to note that in each series one or two cultures survived a dose of 900 milligram-hours and eventually recovered.

Summary.

1. Cultures *in vitro* of the choroid and sclerotic of embryo chicks were exposed for varying periods to gamma rays from radium and were subsequently sub-cultivated every 48 hours.

2. In one series of experiments cultures were exposed to 100 mg. radium (filtered through 0·5 mm. platinum) at a distance of 0·5 cm. In a second series 300 mg. radium was used under the same conditions.

3. When a delayed lethal effect followed exposure to radium, it occurred sooner in those cultures which had been exposed to the greater intensity of irradiation for a shorter time than in those subjected to a lesser intensity for a longer time, the product of milligrammes of radium and hours of exposure being the same in a given comparison.

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Crinkle "A," an Infectious Disease of the Potato.

By Redcliffe N. Salaman, M.D., M.A.

(Communicated by Dr. H. H. Dale, Sec.R.S.—Received November 15, 1929.)

[Plates 1–4.]

Introductory Note.

Owing to the discovery by the author and his former assistant, Dr. R. H. Le Pelley, of the existence of a disease in the Potato, which, though similar in appearance is yet essentially distinct in its reactions from that described by Murphy and known as Crinkle, it seemed desirable to make a close and analytical study of Murphy's Crinkle. It is the result of this investigation which forms the subject of this paper.

As the new crinkle possessed sharply distinctive properties, though clinically a crinkle, it was decided to give it the name of Para-crinkle. A paper on this disease by myself and Dr. Le Pelley will appear directly. The crinkle here studied was that occurring in the variety President, and I have ventured to designate it Crinkle " A " so as to avoid confusion in the not unlikely event of another crinkle being discovered. Crinkles found in the field in the varieties Arran Banner, Arran Comrade, Champion and Irish Chieftain have also been studied and an account of the same is included at the end of the paper.

It has not been thought necessary to make any mention in the text of controls in regard to the various experiments described. There have, however, been full controls in all cases. In particular, every potato plant used as a stock for grafting has been perpetuated by striking and growing on the head of the plant removed at the moment of making the graft.

My thanks are due to my two assistants, Miss C. O'Connor, who has looked after the plants in the hot-house, and Mr. F. M. Cory, who has helped me with the work throughout the season 1929.

All plants were grown in pots in the insect-proof house of the Virus Research Institute, and an average day temperature of 78·6 and night temperature of 51 was maintained between April 7 and August 13. Comparison of the pot growth with that in the open has not been so far made, as all the work here recorded was done between the months of March and November, 1929. In view of the

interest attaching to the new disease Para-crinkle, where the study has extended over a second year, it was thought best not to delay publication in order to make this comparison.

The first to describe and name the disease group "Crinkle," with which we are concerned, was Murphy (1921) who in 1919 differentiated it from ordinary Mosaic, on the one hand, and Curly Dwarf on the other. Quanjer in Holland described at the same time as Murphy a disease in the variety Eigenheimer, which he considered was an intensified form of Mosaic, and which he called *Welvingsziekte*. Both workers have agreed that the disease they were studying is one and the same. Murphy describes the Crinkle plant as follows :—

> "Affected plants are typically bushy, dwarfed specimens of about the same size and form as the low-headed type of Leaf Roll. The compact appearance of Curly Dwarf is absent. The colour is a pale green, but this feature is not marked. The most characteristic symptom is a pronounced and characteristic puckering and downward curving of the leaves. There is no distinct spotting as in mosaic, but diffused, slightly yellowish areas occur all over the foliage. As death approaches, this colour becomes more pronounced and is accompanied by rusty-brown spots, beginning near the tips of the leaves. The foliage is brittle and easily injured. There does not appear to be in normal crinkle any discolouration of the vascular tissue of the leaf or stem, such as occurs in Streak (Orton, 1920). The feature is present sometimes, but it is believed to be a complication. The plants usually live until the end of the season, behaving in this respect more like Leaf Roll or Mosaic than Curly Dwarf."

Quanjer's description (1923) does not differ in any essential particular from Murphy's. Schultz and Folsom (1923 and 1925) consider the "Medium plus Mosaic" of their earlier work to be the same as the Rugose Mosaic which they described in 1923, and both to be identical with Murphy's Crinkle. This identification is, however, complicated by the fact that their Rugose mosaic was found by me (1929) to be a complex, for when grafted to healthy Arran Victory and healthy President respectively, it induced in both of them acute Streak. The fact that streak was thus obtained does not, however, imply that crinkle was not present also, as we shall see later.

Amongst many field workers, and in particular crop inspectors, crinkle is described as mosaic, and ordinary mosaic is either not diagnosed or considered of insufficient importance to merit official recognition ; hence the importance

of crinkle in agriculture greatly exceeds any estimate of its prevalence which is based on the use of its name in the literature.

The sources of infection were kindly provided by Prof. Murphy, who gave me tubers of President, Irish Chieftain, Arran Banner, Arran Comrade, and Champion, all derived from crinkle plants. The descriptive symptoms of crinkle refer particularly to their manifestation on the variety President, which has formed the basis of the majority of the work on this disease. The choice of President was indicated because it is this variety which fails to react to Para-crinkle, and by so doing one hoped to avoid confusion. The variation from the President type in the clinical picture presented by other varieties studied will be noted.

Crinkle "A" in the variety President. Infection by Grafting.

By means of grafting an infected scion to a well-grown and healthy President plant of this late variety, the development of the symptoms can be followed. In such a seasonal infection, stunting of growth and brittleness of stems are late symptoms, which may not develop till the end of the season; whilst the bushy, many-stemmed dwarfed plant, characteristic of crinkle in the field, is not seen until the following season (Plate 1, fig. 1). The lesions in the leaflets are, however, best seen in the early stage of infection. Five such grafts were made, and the average time of incubation before symptoms appeared was 20 days. The first sign of trouble is found in the apical leaves of the new growth arising from the node on the stock stem directly below the attachment of the scion. The veins of the leaflet become prominent because of the withdrawal of chlorophyll in their immediate neighbourhood; a little later these lineal anæmic areas extend from the main veins to the lesser ones; at the junction of such secondary veins with the main one, the anæmic areas become triangular and spread peripherally, forming a more or less well defined pale patch. Similar areas form at the junction of the finer veins further removed from the main ones. The development of these areas is, however, irregular (fig. 2) both in extent and in their disposition on the leaflet. Contemporary with the formation of the pale patches there occurs a shrinkage of the tissues involved and, in consequence, a varying degree of deformity. Whilst such puckering or deformity of the leaf is always present at some stage of the disease, it rarely reaches in intensity that found in Para-crinkle. Accompanying the mottling and deformity there is a very definite waving of the edge of the leaflet; its extent is variable, and, as in the case of the deformity, not generally so well developed as in Para-crinkle.

Another type of lesion, which is very variable and may be entirely wanting, is the formation of minute necroses, which result in black spots, usually at the junction of two finer veins. Fine necrotic streaks may also form on the under side of the leaflet along the veins, and at a later stage similar fine necroses may occur on the stem. Under hot-house conditions a crinkled plant of the variety President tends, towards the end of the season, to recover so far as symptoms are concerned, so that a plant which is still highly infective may show but few symptoms: the patches fade, the deformity becomes less obvious, and the waving of the edge less pronounced. Such plants, however, are still recognisably sick: the leaves are dry, rough, and rather brittle and of a greyish green, whilst the lower leaves may fall and leave the stems more or less bare (fig. 3).

The crinkle of President was conveyed by means of grafting to several other varieties; as their reaction to the virus differed, a description of the variant forms will be given now and will be of value in the consideration of the further reactions of the disease in President itself.

Arran Victory.—Crinkle "A" was induced by graft in this late variety five times: the average incubation period was 26 days. The disease in this variety is always less severe than it is in President, and very markedly less severe than is para-crinkle in Arran Victory. The main difference as displayed clinically between the disease in Arran Victory and President lies in the fact that in Arran Victory, whilst necroses and deformity are rare and never severe, and waving and rugosity of the leaf though present are far less prominent as symptoms, mottling may, on the other hand, be very distinct, and its formation as chlorotic patches extending from the cleared veins to the angles between them is characteristic (fig. 4). In the case of President we have seen how the leaf symptoms tend to become toned down with age; in the case of Arran Victory this happens also, but with a different end result; the mottling becomes faint and very diffuse, whilst the surface of the leaflet becomes glazed (Plate 2, fig. 5). The substance of the leaflet remains fine and relatively soft, the growth of the leaf is not checked so severely as in President, with the consequence that a leaf plucked from a plant of Arran Victory suffering from crinkle later in the season may escape diagnosis by a competent observer who has not had occasion to study the disease closely. Indeed a leaflet with a peculiarly glazed and vivid green surface, with or without a faint mottle, and only gently waved, has come by me to be regarded as indicating the presence of this disease in Arran Victory and in some other varieties also.

Arran Chief.—This maincrop variety has been infected twice by grafting;

the incubation period was 23 days. The reaction of Arran Chief to crinkle " A " is in all respects similar to that of Arran Victory to the same virus. A similar parallel between the reaction of the two varieties to para-crinkle will be described in a later communication.

Arran Comrade.—A second early variety, in its reaction to crinkle " A " as derived from President occupies an intermediate position between that seen in President and that in Arran Victory. Two grafts were made, and the incubation period was 22 days. Clearing of veins, as in the other varieties, is the first symptom, and the further development of this may be limited to a mottling of a diffuse character (fig. 6). No necroses were observed, and no true deformity, although the waving of the edge was in some leaflets very pronounced. Plants of crinkle-infected Arran Comrade were kindly given to us by Prof. Murphy and these displayed a similar reaction, and like the artificially infected (grafted) plants described above, developed at a later stage a glazed appearance very similar to that seen in Arran Chief.

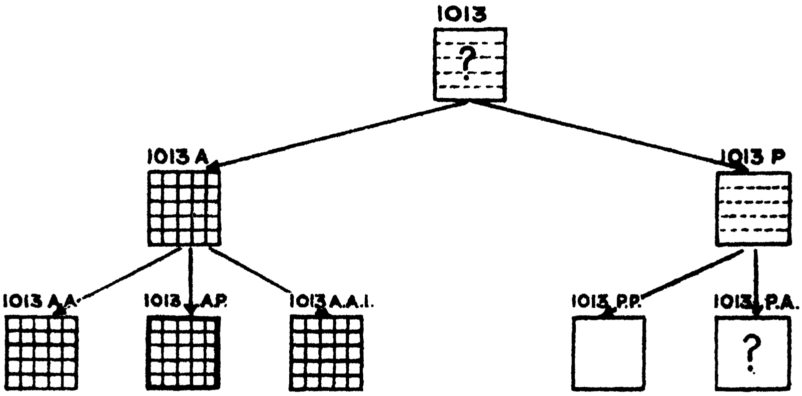
Abundance.—A late variety, was infected with crinkle " A " from President three times, the incubation period was 22 days. The reaction of Abundance is peculiar, in that the resultant symptom complex is not a crinkle at all, but a mosaic which tends to fade away more or less completely. In one of the three infected Abundance plants, no specific symptoms developed, though there was something indefinable about the appearance of the leaves which was noted as " suspicious " ; inoculation of the juice of this plant into Datura produced the characteristic mottling of crinkle " A " which will be described later, showing that the virus was present. Moreover, whereas in the varieties so far considered, the mottling has originated in the immediate neighbourhood of the veins, in this case the pale areas are developed between the veins, and the picture presented is that of an interveinal mosaic (fig. 7) ; it is likewise an interveinal mosaic which is induced in healthy President when grafted with crinkle infected Abundance scions. The infection of Abundance with crinkle (by graft) is not accompanied by any necrosis or deformity and only by a very slight and transient waving.

The peculiarity of the reaction of Abundance to crinkle " A " as well as the subtlety of the relationship between virus and varietal host, is to be seen in the following observation :—

In an isolation plot of the Institute's supposedly virus-free Abundance was noted a plant which was slightly shorter than its very well-grown fellows and whose leaves were a little rugose ; no mottling was observed, and the plant would have been passed by many as healthy. A piece of the plant was removed

and grafted to healthy Arran Victory and President plants. The results of this and subsequent graftings are shown schematically below.

In this case we are presumably dealing with an uncomplicated case of Crinkle "A," though the possibility of Para-crinkle being also present cannot be quite excluded. Abundance itself has suppressed most of the symptoms. Grafted to Arran Victory the disease largely regains its symptomatic expression common in that variety ; on further passage it produces some streak as well as retaining its clinical level. On the other hand, when the Abundance is grafted to



1013. The original Abundance plant with very slight dwarfing and a little ruffling of the leaflets.

1013 P. Grafted to President—stock shows a little blotchy mottling.

1013 P.A. 1013 P. grafted to Arran Victory—stock shows no definite disease but the glazed leaves and pallor suggest rather than demonstrate any disease.

1013 P.P. 1013 P. grafted to President—notwithstanding the vigorous growth of scion and stock, no symptoms supervened.

1013 A. 1013 grafted to Arran Victory producing a mild but definite picture of crinkle "A" in Arran Victory.

1013 A.A. and 1013 A.A.I.,1013 A. grafted to Arran Victory—an exact reproduction of the symptoms of 1013 A.

1013 A.I.P. 1013 A. grafted to President produced a crinkle "A" in the stock with some streak, which latter, however, did not advance.

President the suppression of symptoms becomes more complete, so that mild mosaic or perfectly healthy-looking plants result.

Champion.—An old late variety, is difficult (perhaps impossible) to obtain in an absolutely healthy virus-free condition. With the assistance of Mr. Davidson, of the Free State Department of Agriculture, and Prof. Murphy, the author has selected and raised stocks of this variety which are at least to all appearances healthy, and which have reacted so far as such in all the tests applied. One such Champion plant has been grafted with a scion of crinkle

President. The resultant lesions have been very similar to those obtained in Abundance, viz., the production of an interveinal mosaic. Crinkle, *i.e.*, a symptom complex similar in general to that described in the case of President, occurs in the field on Champion plants ; such plants are being studied in the Institute and so far it is clear that they harbour the true Crinkle "A" virus but whether there is some other virus present also has not been determined.

Sharpe's Express.—This early variety was infected with crinkle from President by grafting twice ; in one case there was no trace of symptoms, and in the other a suspicion of mottling ; but as this did not appear until the plant was maturing, its significance is doubtful.

Di Vernon.—Also an early variety, was infected once in the same manner as the other varieties but developed no morbid symptoms at all. In this case, as indeed in that of Sharpe's Express, although our experimental stocks have been repeatedly tested, we do not feel entirely confident that they are really healthy ; they are, however, symptom-free.

So far, the varieties which have been infected with crinkle from President by grafting have responded by developing a disease which, symptomatically, is either crinkle or a form of mosaic. In the group of varieties next to be considered, the reaction is a different one ; whereas necrotic spots and streaks were only an occasional and indeed rare feature in the earlier series, in this latter it appears as the dominant one, and mosaic mottling which in the former was characteristic, here falls into the background or is entirely wanting.

The variety which demonstrates this reaction at its best is King Edward. Six plants were grafted with scions of President suffering from crinkle "A" ; the average incubation period was 29 days. The first symptoms appear in the apical leaflets of the branch growing from the node nearest to the graft and take the form of small, dark, necrotic spots on the very young leaflets (fig. 8). These grow into irregular angular blotches (Plate 2, fig. 9) which ultimately involve the greater part of the leaf surface, but before this stage is reached the leaf has already begun to wilt and soon hangs lifeless from the stem (Plate 3, fig. 10). Simultaneously with the above, a necrotic destruction of the veins of the leaflets occurs in the form of small streaks which rapidly extend along the veins and generally spread to the main petiole. A day or two later it will be found that the necrotic process has attacked the next lower nodal growing point, to be followed successively by those lower down the stem. The lowest leaves survive the longest but they too fall in the end, and the whole plant dies in about 18 days after the first symptoms appear. The crinkle-affected scion remains unaffected and may be seen still relatively vigorous on the stem of the

stock whilst the leaves of the latter are hanging by threads perpendicularly downwards—a typical picture of Atanasoff's leaf-drop streak. Except in the lowest leaflets, where sometimes mottling may be seen, there are no symptoms recalling either crinkle or mosaic.

The reaction of King Edward to the crinkle of President, so dramatic in its violence no less than its uniformity, cannot, however, be regarded as a simple one. It had already been discovered that King Edward is a carrier of para-crinkle; and our (*i.e.*, the author's and his late assistant Dr. R. Le Pelley's) observations have proved that every stock we have examined of this variety, no matter how healthy its appearance, nor where its place of origin, is affected with para-crinkle. Indeed we seriously doubt whether it is possible to-day to retrieve any stock of King Edward which is free from this latent virus. The leaf-drop streak which results on infection by grafting with President-crinkle, must be regarded as the reaction of the variety not merely to crinkle "A", but to the mixture of crinkle "A" and para-crinkle. Another difficulty is this: ordinary crinkle, *i.e.*, a symptom-complex comparable to that seen in President-crinkle "A," is quite frequently seen in the field stocks of King Edward. I have examined three such distinct stocks, and in each case have produced by grafting on President symptoms of crinkle identical with those of the stock President-crinkle we have been using, and on Arran Victory the typical symptoms of para-crinkle in one case, and of crinkle "A" in the other two. It would appear, therefore, that when King Edward, infected from its origin with the latent para-crinkle, receives in the field the further infection of crinkle "A" presumably by aphides, the disease thus communicated is less severe than that induced by grafting with crinkle derived from President.

Epicure.—An early variety, reacts in a similar way. Two plants were grafted with President-crinkle "A" (fig. 8); the incubation period was 23 days, and in a very short time both plants were killed.

Great Scot, a second early variety, has been grafted seven times with President-crinkle "A"; the average length of the incubation period was 17 days. In three cases the first symptoms were clearing of the veins followed by a mottling of the leaflets of a diffuse type, which subsequently cleared up, leaving either no definite sign of disease or else a peculiar glazing of the surface similar to that which has already been noted in other varieties. This reaction is not, however, the characteristic one exhibited by Great Scot; far commoner is a highly destructive streak.* As in the less severe reaction, clearing of

* My colleague, Dr. Kenneth Smith, has always obtained a streak as the result of infecting Great Scot with the virus of Myatt's Ashleaf crinkle.

veins and a more or less definite mottling appear in about 15 days; in some cases the mottling is bold, formed in the angles of the veins and quickly passes into a necrotic condition (fig. 11). This, accompanied by a certain amount of deformity and waving, gives rise to a picture in which the essential features of crinkle and streak are blended. Later the symptoms develop in a manner similar to that seen in the case of King Edward, and the whole plant succumbs.

There is good reason to believe that our experimental stocks of Great Scot are virus-free, and that one is not dealing with a problem complicated by the presence of other viruses, as in the case of King Edward. Nevertheless, the problem is not quite straightforward, inasmuch as we not only have this alternative reaction but the fact that crinkle in Great Scot in the field is very uncommon. This may mean no more than that the virus which reaches it in the field, not coming *via* President, is not so virulent, or that coming to it *via* aphid inoculation its character is modified. We shall return to this point later.

Infection by Needle Inoculation.

Considerable emphasis has been laid on the fact that the method of communication employed in the experiments so far described has been that of grafting. I have found throughout the very extensive series of grafts made in the last three years, that by grafting a scion from any one variety suffering from a virus disease to a healthy plant of the *same* variety, the result is to reproduce in the latter exactly the same disease, indeed the same grade of it, as was present in the former, always supposing that the union between stock and scion is a good one. Hence any variation between the morbid appearance of the infected plant from that of the source plant, when grafting is the method of communication, must be due to differences inherent in the latter—either morbid or varietal. It has been one's aim to exclude the former and concentrate on the study of the latter. That we are able to deal with virus-free plants in our experiments is due to the activity of the station, whose main object is to produce such, an account of which work it is hoped may be published shortly.*

If instead of infecting healthy experimental plants by grafting, we do the same by means of inoculation, we obtain results which in some cases are distinctly unlike those obtained by grafting. The method of inoculation employed was to grind up a few leaflets of the infected plant in a sterile mortar with a few drops of water, place portions of the juice and débris on to three

* For further consideration of the question of healthy stocks for experimental purposes, see the later paper on Para-crinkle.

leaflets of the experimental plant, and gently rub and prick through the inoculum into the healthy leaflet below with a sterile needle. Following the same order as was done in the grafting, we find:—

Infection of President with President-crinkle "A" by Inoculation.—This was done ten times directly and a great many times indirectly, with an average incubation period of 24 days. In all cases the resultant lesion was the same, viz., a mild mosaic mottling, without the marked clearing of veins so common as a first symptom after grafting; this mottling invariably faded almost entirely away. There was neither deformity of the leaflet, waving of the edge, nor ruffling of the surface. Occasionally the mottling tended to be "blotchy" and reminiscent of crinkle, but it never proceeded beyond the stage seen in fig. 12.

Infection of Arran Victory with President-crinkle "A" by Inoculation.—Seven healthy Arran Victory plants were inoculated directly and many more indirectly, the average incubation period was 18 days. The resultant lesion was again a mild mosaic, varying from a scarcely perceptible mottling to a distinct and even blotchy one, which in all cases inclined to fade. There was neither deformity, waving, nor ruffling of the surface. Fig. 13 (Plate 3) illustrates the more advanced type of lesion and depicts a mosaic of an interveinal type.

Infection of Arran Chief with President-crinkle "A" by Inoculation.—One plant only was infected, the incubation period was 62 days, and the only result was the appearance on a few leaflets of a few minute necrotic spots with some streaks on the lower surface of the veins. The remainder of the plant retained its normal appearance and the lesions did not advance further. There was no mottling. Until this transference is repeated it is impossible to say whether the lesion should be regarded as an incipient attack of streak, or whether this variety is practically unaffected by inoculation. The further question as to whether it is a carrier under these conditions of inoculation remains to be seen. Field experience with this variety tends to support this view.

Infection of Arran Comrade with President-crinkle "A" by Inoculation.—Only one plant was inoculated and that, except for a slight pallor, displayed no symptoms. That it was, however, infected was shown by grafting the Arran Comrade on to a healthy President, which in 17 days displayed an interveinal mosaic throughout all the young leaves, which became more pronounced later, the pale areas spreading from the interveinal position across the veins.

Infection of Abundance with President-crinkle "A" by Inoculation.—One plant only was infected, a faint mottling appeared on the twenty-eighth day, which became more generalised later but not more severe. There was no deformity, waving, or ruffling of the leaflets.

Infection of Champion with President-crinkle "A" by Inoculation.—One plant was infected; after 62 days a very faint mosaic mottling appeared which faded away again.

Infection of Sharpe's Express with President-crinkle "A" by Inoculation.—One plant was infected; after 24 days a definite interveinal mottling appeared in the older leaves, whilst in the younger ones the mottling was interveinal but spotty.

The varieties so far recorded are the same as those contained in the groups which, on grafting, produced crinkle, or, in the case of Abundance, a mosaic. In the next group we have the varieties which, on grafting, produced streak.

Infection of King Edward with President-crinkle "A" by Inoculation.—This infection was repeated five times, the average incubation period was 18 days. One plant remained unaffected. Two plants developed a modified crinkle, *i.e.*, the symptoms began with clearing of veins, followed by mottling, which in places was definitely blotchy and extended from the veins. This was accompanied by a well developed waving of the leaflet edges, and occasional deformity. In one case a few necrotic spots appeared in the younger leaves, and some of the older died of streak. The clinical picture was, on the whole, very similar to the crinkle seen in the field on commercial King Edward plants.

Infection of Great Scot with President-crinkle "A" by Inoculation.—Unfortunately, only one healthy plant could be spared for this experiment, and that survived entirely unharmed; from the rarity of crinkle in the field in this variety such a negative result is to be expected.

Infection of Epicure with President-crinkle "A" by Inoculation.—One plant only was used; after 16 days there was some slight clearing of veins, but this cleared up and the plant remained healthy for a time and then collapsed, apparently with streak. As in the case of Great Scot, such an ambiguous result, despite the rapidly disastrous results of infection by grafting, is not unexpected, for crinkle in the field is rare in Epicure.

The facts recorded show unequivocally that the same virus, introduced by two such different methods as needle inoculation and grafting, produces in the newly infected plant symptom-complexes so distinct that, in accord with our existing knowledge and our present system of nomenclature, we should be bound to give them different names.

In every case grafting produces a more severe disease than needle inoculation (Table I), but the varietal reaction produced by the latter is more in accord with field experience. Just as in the work of my colleague, Kenneth Smith

Table I.—Comparison of results in different varieties of Potato following Infection with Crinkle "A" from President obtained by grafting and needle inoculation respectively.

Variety.	No. of plants.	Incubation period, days.	Result of grafting.	No. of plants.	Incubation period, days.	Result of inoculation.
President	5	20	Crinkle	10	24	Mild mosaic.
Arran Victory	5	26	Crinkle of special type	7	18	Mild interveinal mosaic.
Arran Chief	2	23	Crinkle of type similar to Arran Victory	1	—	Nil.
Arran Comrade	2	22	Very mild crinkle.	1	—	Nil, but proved to be carrying infecton.
Abundance	3	22	Interveinal mosaic	1	28	Mild mosaic.
Champion	1	19	Interveinal mosaic	1	62	Very mild mosaic.
Sharpe's Express	2	19	? Mild mosaic	1	14	Interveinal mosaic.
Di Vernon	1	...	Nil.	—	—	—
King Edward	5	29	Streak	5	18	Crinkle with some lesser symptoms of streak.
Epicure	2	23	Streak	1	16	Clearing of veins. Sudden death.
Great Scot	7	17	Streak	1	—	Nil.

(1929), who has recorded the difference between inoculation of Tobacco with Potato mosaic, when effected by means of aphides, and the far more serious symptoms arising from needle inoculation, certain problems arising from these observations demand consideration. They may be stated thus :—

1. Does the inoculum derived from President-crinkle "A" contain a single and specific virus, or is it a mixture of two or more ?
2. Is the difference of reaction in any given variety induced by needle inoculation of crushed tissue, as compared with grafting of the living President-crinkle scion, due to a selective process, or to the fact that the dosage by grafting is not only infinitely larger than by inoculation but is also continuous and progressive ?

The question of a mixture of viruses being present in President-crinkle remains, so far as our work goes, not only unsolved but scarcely attacked, and until we are in a position to deal in the laboratory with the properties of the

filtered virus we are not likely to make any serious advance in this direction. On the other hand, the constancy of the symptoms of the disease in the variety President would suggest that either crinkle is a unit and specific virus, or that it is a balanced and uniform mixture. Whilst virus diseases are identified solely on a clinical basis, it is impossible to answer this and other urgent questions. No potato disease is more clearly characterised or more uniform in its expression than streak, and yet we have in President-crinkle a virus which will produce streak in several varieties though not in President itself. On the other hand. we have on many occasions extracted from apparently healthy Up-to-date and Duke of York a virus which kills President promptly with "streak." Obviously streak is a symptom-complex produced by more than one virus.

We have, however, one piece of evidence which points towards President-crinkle being a mixture of viruses. As we shall see later, passage of the virus through *Datura stramonium* constantly modifies, in the direction of reduction. the severity of the symptoms produced on reinfecting the potato; instead of crinkle one gets mosaic, and instead of a severe mosaic, a mosaic with the mildest symptoms. Now we saw that President-crinkle "A" when grafted or inoculated to Epicure produced in the latter streak; if the virus be passed through Datura by inoculation and then brought back by grafting to President or Arran Victory and again introduced, whether by grafting or inoculation, to Epicure, the result is a lethal "streak." By inoculating or grafting from the same source into other varieties, various reduced forms of mosaic arise, but in the case of the pathogene which affects Epicure, no such modification has taken place, and one would appear to be justified in assuming a mixture in which one virus or one set of viruses is modified by *passage*, and another, viz., that producing streak in Epicure, is not. That Datura may further act in a selective manner in the presence of two or more viruses we shall see to be true in the case of crinkle and para-crinkle, when the latter is excluded and the former accepted on inoculation. The evidence, though incomplete, points to the answer to question 1 being that President-crinkle contains a mixture of viruses.

Healthy President was inoculated ten times with President-crinkle juice with the production in each case of a mosaic. Further inoculations and graftings were made from these latter as shown in Table II.

Three healthy President and three healthy Arran Victory plants inoculated with the mosaic, induced by inoculating instead of grafting President-crinkle, responded in their turn by producing in each case a simple mosaic. When

Table II.

Description of source.	Condition of source.	Inoculated.	Grafted.	Stock.	Pot No. of stock.	Condition of stock.	No. of Datura plants.	Reaction produced by inoculation of source into Datura.
Healthy President inoculated with President-crinkle	Mosaic		—	President	248 P1, P4	Mosaic	—	—
,, ,, ,, ,,	,,	+		,,	248 P1, P1	,,	5	Similar to that produced by President-crinkle itself.
,, ,, ,, ,,	,,	+		,,	248 P1, P2	,,	5	,, ,, ,,
,, ,, ,, ,,	,,	+		,,	248 P1, P3	,,	5	,, ,, ,,
,, ,, ,, ,,	,,	+		Arran Victory	248 P1, A1	,,	—	—
,, ,, ,, ,,	,,	+		,,	248 P1, A2	,,	—	—
,, ,, ,, ,,	,,	+		,,	248 P1, A3	,,	—	—
,, ,, ,, ,,	,,	—	—	—	—	—	5	Similar to that produced by President-crinkle itself.
,, ,, ,, ,,	,,	—	—	—	—	—	5	,, ,, ,,
,, ,, ,, ,,	,,	—	—	—	—	—	5	,, ,, ,,
Healthy President inoculated with Irish Chieftain-crinkle	? Healthy		+	President	244 P2, P	Mosaic	—	—
Sharpe's Express inoculated with Irish Chieftain-crinkle	Very faint mosaic		+	,,	244 S.E.P.	,,	—	—
Healthy President inoculated with Arran Comrade-crinkle	Faint mosaic		+	,,	246 P1, P.	,,	—	—
Healthy Arran Victory inoculated with Arran Comrade-crinkle	Mosaic		+	Arran Victory	246 P1, A	,,	—	—
Healthy Arran Victory inoculated with President-crinkle	,,	+	—	—	250 A.4,	—	5	Similar to that produced by President-crinkle itself.
,, ,, ,, ,,	,,	—	—	—	250 A5,	—	7	,, ,, ,,
,, ,, ,, ,,	,,	—	—	—	248 A2.	—	4	,, ,, ,,
Healthy Arran Victory grafted with President-crinkle	Crinkle	—	—	—	248 A.2	—	1 (grafted)	,, ,, ,,

grafting was employed the reaction was the same—a simple mosaic. Whatever change, therefore, the original needle inoculation produces is maintained and fixed. The reaction in Datura, however, retains the characters of that produced by inoculation of the original President-crinkle juice and, as we shall see, differs from that resulting either from an original mild mosaic or from para-crinkle. It would seem therefore that whilst the virus present in these plants is essentially the same as that in President-crinkle "A" itself, it produces a permanently modified reaction in the healthy President or Victory plants. Similar results obtain (see Table II) when instead of crinkle in President the crinkle of Irish Chieftain or Arran Comrade is used.

It is suggested that this modification is due to the smallness of the dose which ensues from needle inoculation, and that the mosaic which it produces must be looked on as a mild attack of the original disease crinkle. This view receives further support from the fact that on several occasions we have found an exactly similar difference of reaction as exists between grafting and inoculation to follow on grafting, when for some reason the scion has failed to make a satisfactory union. Such a scion may persist in a flagging condition for weeks without any real union.

A third method of inoculation was carried out by my assistant Mr. F. M. Cory. Crinkle "A" juice derived from crushed up leaves was diluted with an equal volume of water, the tops of healthy Arran Victory plants were placed in tubes containing the juice and kept there 4 days, at the end of which time they had imbibed most of the fluid. These tops were then grafted back to their original stocks of healthy Arran Victory. Two such experiments were carried out; in both cases a mild mosaic without waving or deformity developed on both scion and stock. There was no sign of crinkle, not even a mosaic, which on any individual leaf could be described as severe. In both cases the scion grew after grafting from 4 inches to 18 inches, so that union must have been good. The difference between these infections and one by direct grafting from a crinkle plant is solely one of the method of infection, of how the virus first reached the healthy plant. It is this matter of method which appears to play an important part in the symptomology of the virus-diseased potato plant.

It is well known that some viruses are capable of a very high dilution, whilst retaining their full infectivity. Notably is this so in the case of tobacco and tomato. Johnson (1928), however, has shown that in regard to rugose-mosaic of the potato, which is presumably the same as crinkle "A," a dilution of 1-10 is sufficient to inhibit. Between full infectivity and inhibition, may not an intermediate infectivity, clinically represented by mosaic, result?

It may be argued that according to the dosage hypothesis the crinkle symptoms should supervene, inasmuch as the crinkle virus has been introduced, in no matter how small a quantity; yet once in, it must presumably increase in quantity with the growth of the plant, for the latter may grow exceedingly. The fact that this does not occur, and that grafting from the plant with the modified disease does not induce crinkle but only the modified mosaic in a healthy plant, points to some definite attenuation of the virus having taken place when it was first introduced (by the needle) into the plant as a result of the minimal initial dose.

Kenneth Smith* has shown that a single infected aphis, feeding for so short a time as 2 hours on a healthy tobacco or potato plant, suffices to convey the infection of some of the virus diseases he has studied, as readily as do a batch of 18 aphides. The dose conveyed by the aphis must be excessively small, even when compared to that which an ordinary needle inoculation affords, yet in the case of Myatt's Ashleaf, Kenneth Smith has found crinkle conveyed in all its severity by a few aphides to a healthy plant. These facts, however, do not necessarily controvert the suggestion made that the difference of results between needle and graft inoculation is primarily one of quantity.

The aphis inserts the virus by means of its proboscis straight into the phloem vessels; there is neither waste of inoculum nor destruction of tissue as in needling. It is hoped next season to infect plants by means of very fine capillary tubes and eliminate the latter element. Again, we have no idea what happens to the virus, or the plant juice in which it exists, within the body of the aphis. There may well be a separation, *i.e.*, a concentration of the virus which, together with the perfect method of inoculation, allows the aphis to work with doses vastly smaller than that practical in such a gross operation as needling.

The reaction produced by the virus of President-crinkle on Datura and Tobacco, and the further reaction of these two species on the character of the virus, throw some light on this question of virus-modification, and will now be considered.

The Reaction of Healthy Datura Plants to Crinkle " A."

When an infected scion of President or Arran Victory is grafted on to a young Datura plant of about 6 inches in height, it is necessary to behead the latter and before the appearance of symptoms to await the growth of leaves from the node below. This union was effected successfully five times. The

* Communicated verbally.

Datura plant never regained the size of the controls, the new leaves remained small and lanceolate (fig. 14), rarely showing any tendency to adopt the normal crenate border. After a period of about 18 days, the veins of the young leaves show a definite clearing, and the leaf itself becomes ruffled and mottled, the latter developing later into yellow patches of variable but relatively large size. Throughout the remainder of the leaf the green colour becomes paler; the ruffling of the leaf may pass into a definite puckering and deformity, but this is not common.

If instead of using crinkle "A" from President direct, we pass it by grafting through Arran Victory, and use this as scion, then the reaction in the Datura is less violent, and the effect in the leaves is identical with that described below as a consequence of needle inoculation. This was done twice, each time successfully.

If instead of grafting, the Datura be infected by needling with the crushed juice of President-crinkle, the result is markedly different. The growth of the Datura plant, if it be 4 to 6 inches high at the time of inoculation, is only slightly checked, but if it is inoculated whilst the seedling plant is still only furnished with the cotyledonary leaves—a highly practical method—then the resultant plant lags considerably behind the control. The Datura leaves grow to their usual size and acquire the adult shape.

President-crinkle was inoculated directly into nine Datura plants, and the same virus after *passage* through other potatoes (and occasionally through both Datura and Tobacco) was inoculated into several scores of healthy Datura, with a constant result (Table III). Infection occurs in about 90 per cent. of the plants, and symptoms develop on an average in 13 days. The first and most constant symptom is a clearing of the veins, causing them to stand out boldly against the dark green background of the leaf tissue. This is followed by a mottling which usually commences at the base of the leaf (fig. 15). The earliest mottling may be strictly speaking not a true mottle, for it is often in the form of a very fine network occasioned by the "clearing" of the finest ramifications of the veins, which produces a picture of a fine network on a dark background. This stage soon passes; the smaller enclosed areas themselves become pale by reason of the coalescence of the "clearings" round the very fine terminal veins. In this manner a much coarser meshed mottling results, in which the larger cleared veins now act as boundaries, and the coalesced finer clearings, with a varying amount of spread into the tissues around, form the main mass of the pale tissue enclosed.

There is now to be seen a feature which is more or less characteristic of

the reaction of crinkle "A" and its allied diseases. Along the margins of the cleared veins there persists a fine line of intensely green tissue, so that the bright cleared vein is picked out by a dark border on either side. If the infection is severe, this larger mesh now breaks down, the pale areas tend to coalesce, and in doing so the whole pattern becomes interveinal, and many of the dark borderings to the veins are merged into the larger pale area, with the consequence that some of the larger veins in the same leaf may be bordered by dark green lines, whilst others are lying in unpigmented pale yellowish areas (fig. 16).

This type of reaction in Datura appears in our experiments to be a constant and distinctive reaction of the crinkle "A" virus, in contrast to the reaction given by para-crinkle and simple mosaic of a non-crinkle origin. Interveinal mosaic, which appears to have a special relation to crinkle "A," gives a similar reaction in Datura to that produced by crinkle "A." As the Datura plants grow older, the crinkle mottling may gradually disappear in the older leaves; removal of a portion of the growing parts generally results in a new growth of leaves with the characteristic mottling.

The infection was next conveyed by needle from infected Daturas to a second series of healthy Datura plants, ten times, with 100 per cent. success. The reactions have precisely the same character, the same variation in intensity on any one plant, as was observed in the first passage of the virus to Datura. There is no alteration in virulence.

The form of crinkle found in the field in the varieties Irish Chieftain, Arran Banner, Arran Comrade and Champion, all give the same reaction in Datura as does President-crinkle, and here also a second passage through Datura does not affect the character of the reaction. For details of these infections see Table III.

One interesting departure from the normal crinkle "A" reaction occurred with an inoculation on Datura from the mild mosaic occasioned in Abundance as a result of grafting President-crinkle on to that variety. Here the clearing of veins was less marked (Plate 4, fig. 17), the pallor began in the centres of the interveinal areas as a very fine punctate marking which later took the form of small rings. The dark bordering to the main veins characteristic of crinkle "A" is present here, but is not so noticeable because these borders are themselves so extensive as to fill in the major part of the interveinal areas. Abundance, of all the varieties so far worked with, has produced the most distinct modification of the virus in its further reaction on the Potato; it is of interest that on Datura its reaction should likewise be distinctive.

Table III.

Source.	No. of Datura inoculated.	No. of Datura grafted.	No. of successful infection.	Incubation period. Days.	Pot No. of the Datura.	Type of reaction.
President crinkle	5	—	4	10	250 Dat.	Mottling with dark lines along edge of the veins.
,,	5	—	5	10	248 Dat.	
,,	4	—	3	27	321 Dat.	
President crinkle passed through Arran Victory	4	—	4	13	248 A.2, Dat.	
,, ,, ,, ,,	5	—	3	17	250 A.2, Dat.	
Irish Chieftain crinkle	10	—	10	8	244 Dat.	
Arran Banner crinkle	7	—	5	16	245 Dat.	
,,	—	1	1	18	245 Dat. 1*	
Arran-Comrade crinkle	5	—	5	10	246 Dat.	
,,	—	1	1	18	246 Dat. 1*	
Champion crinkle	5	—	3	12	247 Dat.	
Myatt's Ashleaf crinkle	25	—	24	8	819-823 Dat.	
Datura infected with President crinkle	5	—	5	11	250 Dat., Dat.	
,, ,,	5	—	5	13	321 Dat., Dat.	
,, Irish Chieftain crinkle	5	—	5	16	244 Dat. 1, Dat. 2	
,, ,,	—	1	1	16	244 Dat. 1, Dat. 1	
	93		84			

Average incubation period = 13 days.
Successful infections = 90 per cent.

* Symptoms similar to those when President crinkle "A" is *grafted* to Datura.

Table IV.

Source.	Method of infection.	No. of plants used.	No. of successful infections.	Incubation period Days.	Variety of Potato.	Pot No.	Result.
Datura grafted with President crinkle	Graft	2	1	26	President	850 4P.	Mosaic.
,, ,, ,,	Inoculation	1	0 ?	?	,, ...	850 4P.1	? Healthy.
Datura inoculated with President crinkle	Graft	1	1	17	Arran Victory	399	Mosaic.
,, ,, ,,	,, ...	1	1	17	President	401	,,
,, ,, ,,	Inoculated....	5	3	18	,, ...	250 Dat. P.1, P.2, and P.3	Very mild mosaic.
,, ,, ,,	,, ...	5	1	13	Arran Victory	250 Dat. A.1	Mosaic.

The reaction of Datura to interveinal mosaic as occurring in President, in Eigenheimer and in Arran Victory (to name only the three varieties in which it has been tested) is of precisely the same nature as that found in the case of crinkle "A."

The reaction of simple mosaic—as apart from the mosaics induced as a sequel of crinkle needle inoculation and Datura crinkle modification—was tested out in one variety only, viz., Arran Victory. The stock was the same as has been used by Kenneth Smith in his Potato mosaic experiments. In all, 4 separate sources of Arran Victory mosaic were inoculated by needle into 17 Datura plants; 3 failed to make a visible reaction, in 8 the veins of the Datura leaves were cleared and a suspicion of mottling was observed, and in 6 there was a definite mottling. This mottling, which begins at the base of the leaf as a clearing of main veins, extends from thence on either side of the veins (Plate 4, fig. 18).

The pallor, never very bright, may then appear on the finer veins, and coalescing with neighbouring affected parts, there is produced a definite meshwork type of mottling. These areas in their extension may approach a main vein before a change has taken place locally in its immediate neighbourhood. In such a case one may find a pale area bordered by a dark green line lying alongside a vein, but this is occasional only on any one leaf. The picture produced is quite distinct from that induced by crinkle "A" with its characteristic dark green lines bordering the veins.

The reaction of Datura to para-crinkle is so distinct and differs so completely from that of crinkle "A" that its consideration may be left to the communication dealing with that disease.

Infection of Healthy Potato Plants with the Crinkle "A" Virus after Passage through Infected Datura.

Daturas infected by grafting when used as scions and grafted to healthy Potato plants, produce in the latter a mild mosaic. Daturas infected by means of needle inoculation and grafted to healthy Potatoes, do the same. Daturas infected by inoculation and themselves used as the source of infection for needle inoculation into healthy Potatoes produce in the latter either no reaction or a very mild mosaic. Details of the experiments are shown in Table IV.

The effect of passing the crinkle "A" virus through Datura is clearly to so alter it that on introducing it again into the Potato, it produces a far less severe disease than that of the original source. We obtain a mosaic, generally of a very mild form, instead of a crinkle. This reduction of the virulence varies

in degree with the method of inoculation employed. A series of preliminary trials were made with a view to discover whether this change of virulence was maintained on passage to other varieties and to obtain some light on the nature of the change in the virus, whether it was in the nature of a selection or suppression of one or more of a mixed group of viruses, or whether it was essentially a modification of a unit virus responsible for crinkle "A."

The crinkle-infected Datura was grafted to healthy Arran Victory, producing a condition which at first might be called a severe mosaic but later showed only a mild mosaic without deformity, waving or appreciable ruffling. This plant, whose pot No. was 399, was used as a source of infection both by grafting and needle inoculation to a number of varieties, with the results shown in Table V. A further graft of inoculated Datura to healthy President is represented by pot No. 401 ; like 399 it exhibited a rather mild mosaic.

Reviewing Table V we see that, leaving Epicure and Arran Crest to one side, the reactions in the remaining varieties are a mosaic and not a crinkle ; and further, that where the infection was by needle, the mosaic is definitely a mild one. In two cases, 399 A.3 and 399 P.3, after the mild mosaic resulting from needle inoculation was established, the plants were grafted with scions from 399, the source of the needle inoculum. The result of this additional infection was to slightly increase the symptoms in the two plants as compared with those of the controls 399 A.V., 399 A.V.2, 399 P.1, and 399 P.2. New growth after the grafting displayed a rather lighter and more blotchy mottling, but in both cases the symptoms receded again to those of an ordinary mild mosaic. At no time did the clinical picture coincide with that of crinkle. There was no appreciable difference between the intensity of reaction in these two plants and those present in 399 A.4 and 399 P.4, in which the infection was conveyed by grafting alone. The reaction of Tobaccos to the virus as modified by Datura is a reduction of symptoms, if anything more pronounced than that in the Potato.

The very different reaction produced by the two varieties Arran Crest and Epicure to direct infection of President-crinkle "A" has already been referred to. Here again, after passage through Datura, there is no essential difference, whether infection is produced by needle or by graft ; in both there results a lethal streak, a little more rapid in the case of the graft. Finally, passage of the 399 stock into further Daturas merely reproduces the crinkle "A" symptoms of Datura infection, slightly reduced but not altered in character.

We have therefore evidence that whilst the passage of crinkle "A" virus through Datura markedly modifies its crinkle-producing properties, it has no

Table V.

Source.	Graft.	Inoculation.	Variety of stock.	Pot No.	Result.
Potato grafted with Datura which was inoculated with President-crinkle " A " — Pot Nos. 399 and 401.	+		President	399 P.4	Mosaic (mild).
		+	,,	399 P.1	,, ,,
		+	,,	399 P.2	,, ,,
	+	+	,,	399 P.3	Mosaic.
	+		Arran Victory	399 A.4	,,
		+	,,	399 A.1	Mosaic (mild).
		+	,,	399 A.2	,, ,,
	+	+	,,	399 A.3	Mosaic.
	+		Abundance	399 Ab.	,,
	+		,,	399 Ab.1	Mosaic (mild).
		+	Arran Comrade	399 A. Cde.	,, ,,
	+		Sharpe's Express	151	Mosaic.
		+	,,	399 S.E.	Mosaic (mild).
		+	Arran Crest	399 A. Ct.	Acute streak.
		+	Epicure	399 E.	Streak.
	+		,,	399 E.1	? Healthy.
	+		,,	399 E.2	Streak.
		+	Arran Victory	401 A.	Mosaic (mild).
		+	Datura (5 plants)	399 Dat.	Reaction same as that of crinkle " A," but less severe.
		+	Tobacco (6 plants)	—	4 plants showed a very faint mottling. Reaction much reduced as compared with crinkle " A " direct.

action on that which in certain specific varieties produces streak, or in Datura produces the characteristic green-line mottle. This would point to the virus of crinkle " A " being a mixture.

We are not at present in a position to state what precisely this mixture is. We have evidence of a streak-producing factor and a crinkle-mosaic factor. As regards this latter, it has been found that when President crinkle " A " is grafted to the variety Abundance, the resulting mosaic was interveinal in distribution, and that when this was conveyed to President it also gave rise to an interveinal mosaic, and this latter when inoculated into Datura gave rise to a modification of the dark line mottling. Crinkle " A " mottling is reproduced exactly by such stocks of interveinal mosaic as were found in the varieties President and Eigenheimer in the field. A similar interveinal mosaic was found by grafting President crinkle " A " into Champion, and by needle inoculation into Arran Victory. It would seem therefore that there is an intimate relation between interveinal mosaic and crinkle " A," which will need further research for its elucidation.

So far the modifications induced by passage through Datura appear to be precisely of the same character as those produced by needling, but if we were right in ascribing the modification produced by the latter to the effect of dosage, the following experiments demonstrate that this explanation fails in the case of Datura.

Double Grafts.

These experiments were devised with a view to determine whether Datura modified the virus or selected certain constituent parts of it. A healthy Potato stock of Arran Chief or President was grafted with a piece of solid Datura stem 2½ inches long and ¼ inch to ½ inch in diameter. This latter was cut out from Datura plants about 12 inches high and was taken from between the nodes so that it had no leaves or buds attached to it, nor at any stage of the experiment did such grow from it. This solid column of tissue, grafted at its proximal end to the healthy Potato stock, was then grafted at its distal end with a scion of President crinkle. Generally the two graftings were done at one time, which, if great care is taken to keep the plant moist and shaded, succeeds admirably ; in a few cases the Datura was grafted first to the stock, and after an interval of about 2 weeks the crinkle scion was added. The result is the same in either case. After a rather variable interval there appears in the stock leaflets evidence of true crinkle which develops as fully as if there had been no intervening piece of Datura stem.

So far then, it would appear that all that virus content which goes to make crinkle "A" disease in President passes unaltered down the solid mass of Datura stem, permeating a mass of Datura tissue about 7 to 10 c.c. and weighing as many grammes. There has been no selective process. Let now the experiment be varied by using a piece of Datura stem which contains in its middle a node and a pair or more of leaves; the grafting follows as before, but here it is better to do it in two stages at a fortnight's interval. Again after a variable interval symptoms appear in the healthy stock Potato plant, but not of crinkle; the most that occurs is a mild mosaic. The only difference between the two experiments is the presence of a few leaves borne half-way down the Datura graft and not weighing more than at most 10 per cent. of the whole Datura tissue.

The leaves, centres of metabolic activity, pass into the stem their resultant products, which here must meet the plant juices of the scion containing the virus or viruses present which emanate from its crinkle leaflets. One can but assume that some interaction takes place in the Datura stem beneath the level of the leaf-bearing node, and that as a consequence the virus which passes on to the healthy Potato stock below is so modified as to be incapable of producing a crinkle as before, but is only able to induce such changes which materialise as a mosaic in the stock plant.

In Table VI details of these experiments are given.

It may be assumed therefore that :—

(*a*) The virus of crinkle "A" is modified during its passage through Datura.

(*b*) That neither needle inoculation nor grafting brings about further modification of crinkle "A" in the Potato after the passage of the latter through Datura. Such differences as do occur between the clinical picture induced by graft and that by needle are small in range, and not, as was found before the passage through Datura, equivalent to a different symptom-complex requiring a distinctive clinical designation.

(*c*) That the modification of the virus, following its passage through Datura, is determined by the interaction of the virus with some physiological product of the metabolism of actively growing Datura leaves. The virus is not modified by the juice existing in the solid Datura stem in the total absence of leaves, nor presumably do the Datura tissues select or withhold any part of the virus.

(*d*) The constituent of the virus in crinkle "A" which produces streak in Epicure is unaltered by passage through Datura.

Table VI.

Pot No. of experiment.	Stock plant.	Date 1st graft.	Date 2nd graft.	Datura stem with no leaves.	Datura stem with leaves.	Scion and source of virus.	Symptoms produced in stock.
250 Dat. 2, P.1	President	10.7.29	10.7.29	+		Crinkle in President	Severe mosaic.
		(union	poor)				
250 Dat. 2, A.1	Arran Victory	10.7.29	10.7.29	+		,, ...	Crinkle.
249 A. Dat. P.	President	10.7.29	10.7.29	+		Crinkle in Arran Victory from President....	,,
249 Dat. A.	Arran Victory	10.7.29	10.7.29	+		Crinkle in President	,,
229 Dat. A.3	,, ...	5.4.28	23.5.29		+	,, ...	Mosaic.
217 A.	,, ...	22.3.29	2.5.29		+	,, ...	Mild mosaic.
250 Dat. P.	President	17.7.29	27.8.29		+	,, ...	Very mild mosaic.
248 Dat. A.	Arran Victory	16.7.29	15.8.29		+	,, ...	Faint mosaic.
249 A. Dat. A.	,, ...	16.7.29	15.8.29		+	Crinkle in Arran Victory from President	No symptoms.

(*e*) The constituent of the virus in crinkle "A" which causes the dark line mottling in Datura is unaltered by passage through Datura, is similar to that producing interveinal mosaic in certain varieties, and is different to that which produces simple mosaic in Arran Victory.

The Reaction of Healthy Tobacco Plants to Crinkle "A" in President.

Reasons of convenience, as well as sound practice, dictate that Tobacco seedlings which are destined for infection must be young and actively growing. Inoculation may be made successfully when the diameter of the whole plant is not more than ½ inch, but it is more practical to use plants about 2 inches in diameter. The reaction of young growing plants is more or less certain and constant—provided always they be kept in active growth at a temperature of at least 70° Fahrenheit. If old plants are used, the reaction is very inconstant; sometimes the plant shows no reaction, whilst at others it may throw up a new leaf bearing vivid mottling. The variety of Tobacco used is also of importance; where not otherwise stated, the White Burley variety has been used. Another, "Virginia," was tried, but found to be less sensitive.

As grafts cannot be made except on reasonably well-grown plants, this method of infection has not been made use of except on one occasion. The variety was Viriginia and the reaction a mottling spreading from the veins and much less intense than that found on young seedlings as a result of needle inoculation. Fifteen White Burley seedlings were inoculated from three different President crinkle "A" sources with seven successes. The symptoms were variable; when most developed there was seen clearing of veins, formation of irregular rings with dark green centres, and irregular mottling of the tissues between any two main lateral veins (fig. 19). When least developed, there was nothing but a faint mottle present.

Passage of the virus through Tobacco to Tobacco was attempted 6 times with success in the second generation, and 12 times with but one failure in the third generation. In both cases the variety White Burley was used. There was no increase whatever of symptoms, indeed some of the original inoculations of the first generation produced a more vigorous reaction than any did in the third. The virus was passed from Tobacco, both in the first and second generation, to Potato as shown in Table VII.

The mild mosaic in the Potato resulting from the passage of crinkle "A" through Tobacco was passed on by graft to a healthy President (250 Tob. 2, P.2P), and to a healthy Arran Victory (250 Tob. 2, P2. A) plant respectively; in both cases nothing but a mild mosaic was produced.

It will be seen that passage of crinkle "A" through Tobacco weakens its

Table VII.

Pot No. of source of inoculation.	Variety of Potato infected.	Pot No.	Result.
250 Tob. 1	Arran Victory	250 Tob. 1, A.1	Mosaic mild with few fine necrotic spots.
,,	,,	250 Tob. 1. A.2	
,,	,,	250 Tob. 1, A.3	
,,	President	250 Tob. 1, P.1	Mosaic mild.
,,	,,	250 Tob. 1, P.2	Mosaic severe.
,,	,,	250 Tob. 1, P.3	Mosaic with few fine necrotic spots.
248 Tob. 4, Tob. 1	Arran Victory	250 Tob., Tob. A.1	Mosaic very mild.
,,	,,	250 Tob., Tob. A.2	
,,	,,	250 Tob., Tob. A.3	
,,	President	250 Tob., Tob. P.1	
,,	,,	250 Tob., Tob. P.2	
,,	,,	250 Tob., Tob. P.3	

reaction on the Potato, and that a second passage through Tobacco still further reduces it. In this respect crinkle " A " differs *in toto* from the virus found in Arran Victory mosaic, which Kenneth Smith has employed in his exhaustive researches on Ring-spot mosaic. Now this latter virus is the same as that which gave the reaction in Datura for simple mosaic referred to on p. 70, and the Tobacco reaction of crinkle " A " is a further piece of evidence that the mosaic derived from crinkle, and that found in Arran Victory in the field, are produced by different viruses.

It is highly probable that many of the simple mosaics met with in the field are in reality crinkles, modified by passage through resistant varieties, as for example we have seen is the case in Abundance (see p. 73). Thus a very mild mosaic is extremely common in even the very best Scotch field stocks of Kerr's Pink. Four such stocks were examined by grafting on to healthy plants of Arran Victory and President respectively ; a simple and mild mosaic was produced in each case, but when juice from one of the Kerr's Pink stocks was inoculated into Datura plants, it produced the crinkle " A " reaction. No direct experiments were made with Kerr's Pink, because the writer up till late in the season had failed to find a single stock of this variety which was not affected with what looks like a mild mosaic, and therefore lacked a stock sufficiently reliable for use in this research.

Crinkle Disease of Arran Banner, Arran Comrade, Champion, and Irish Chieftain.

Specimens of each of these varieties affected with crinkle and obtained from field cultures, were given to the writer by Prof. Paul Murphy. Preliminary testing with grafts on Arran Victory and President demonstrated that they

were all suffering from crinkle "A." A brief sketch will be given of their reactions.

Arran Banner.—The crinkle symptoms are not severe (fig. 20). The leaves are of normal size, rather thin, and tend to become glazed. The clinical picture resembles that of crinkle "A" in Arran Victory. Grafts were made into healthy President plants and produced in them a rather weak form of crinkle; the scions, however, died early. Two grafts into Arran Victory produced in the latter a rather weak form of crinkle. Inoculation to Datura produced the typical dark line reaction of crinkle "A."

Arran Comrade.—The symptoms of crinkle "A" in this variety are very similar to those in Arran Banner and Arran Victory. Introduced by grafting to President, it caused a reduced type of crinkle, whilst on Arran Victory by graft the reaction was identical with that on Arran Victory produced by direct grafting from President-crinkle "A." On Epicure it induced a very mild and transient mosaic.

Inoculation of Arran Comrade crinkle into President produced a mosaic only, and this introduced afresh to President and Arran Victory by grafts again produced only a mild mosaic. Inoculation to Datura produced the typical crinkle "A" mottling, and when the leaves were cut back they threw up fresh leaves similarly mottled. Inoculation to Tobacco produced a rather feebly developed mottling with fine rings.

Champion.—Crinkle in this variety is in appearance more closely related to that in President than the other varieties just dealt with. Grafted to President, it reproduces the full picture of a severe attack of crinkle "A" in that variety; in Arran Victory likewise, the effect is that of a severe attack, with the peculiarities generally seen in this variety with crinkle "A," that is, little deformity and no necroses. Inoculation into Datura produced the dark line reaction of crinkle "A."

Irish Chieftain (fig. 21).—The crinkle of this variety is so outstanding in the brilliance of its mottling and the severity of its deformities, that a more complete study was made of this type than of the others dealt with in this part of the paper. Grafted to three healthy Presidents, it produced the full crinkle "A" reaction of that variety, and into three healthy Arran Victory it did the same. Grafted to Arran Chief the reaction which ensues is similar to that produced by President crinkle in Arran Chief and itself very similar to the reaction seen in Arran Victory. Grafted into healthy Champion the result was exactly the same as the crinkle in the field. Grafted to Abundance it produced no effect, but a graft from the Abundance to President caused a mild mosaic in the latter.

Three healthy Great Scots were grafted with Irish Chieftain crinkle; in all an early type of crinkle appeared which rapidly developed till an acute streak stayed its progress and killed the plants. This behaviour is exactly the same as that of Great Scot when grafted with President crinkle " A." King Edward again followed the President crinkle analogy and died with streak after grafting.

Inoculation of the juice of Irish Chieftain crinkle into plants of the following varieties, President, Arran Victory and Sharpe's Express, produced mild mosaic in all. The mosaics so produced, in the cases of President and Sharpe's Express, were conveyed by grafts to fresh healthy Presidents, producing in these a mosaic of a mild type. An inoculation into a rather weakly plant of Epicure was without effect, though, on the analogy of the similarity of the virus with that of President crinkle, a streak would have been expected to ensue.

On Datura, inoculation of Champion crinkle produced the typical crinkle " A " effect, and the passage through further Daturas, both by inoculation and by grafting, did not alter the intensity of the symptoms. Into Tobaccos of the White Burley variety, a rather feeble mottling developed, accompanied in some by the formation of rings. A preliminary examination of the reaction of a crinkle in the variety Myatt's Ashleaf, indicates that it also is of the same type as those just considered.

It would appear that the disease present in all these varieties is that produced by the virus of crinkle " A." The exhibition of symptoms varies with the variety. The behaviour of Arran Banner and Arran Chief is practically the same as that of Arran Victory when artificially infected from President; whilst the appearance no less than the exact degree of virulence of the crinkle of Irish Chieftain, here investigated at some length, shows that it is very similar in kind and degree with that found in President.

Attention once more is drawn to the reaction of the variety Abundance to crinkle " A," and that of Kerr's Pink mosaic to Datura. They both point to the possibility of the modification of the virus occurring in the field and give support to the writer's views, which will be expounded elsewhere, as to the desirability of maintaining adequate isolation between different varieties when grown in the garden or field.

Summary and Conclusions.

The Potato disease crinkle as defined by Murphy and others, and here called crinkle " A," is described as found on the variety President. The relations of crinkle " A " to para-crinkle are reserved for a further communication.

The transference of the disease crinkle "A" by grafting to a number of different varieties is described; in most, the clinical appearance is that of a crinkle, in some it is acute streak, and in one, Abundance, a mosaic only.

Varietal reaction to infection by grafting with crinkle "A" is not a simple phenomenon; there is evidence that the virus itself is modified, as well as the clinical expression of symptoms on *passage* from one variety, *e.g.*, President, to another, such as Abundance.

The result of infecting various varieties of healthy Potato with crinkle "A" by means of needle inoculation is described. It was found that in those cases where grafting had produced a crinkle, needling gave a mosaic; where grafting caused streak, needling gave crinkle; except in the case of Epicure where streak reappeared, and, finally, where grafting had produced a mosaic, needling did the same, but of a less severe character.

The possible cause of the distinction between needle inoculation and grafting is discussed, and the suggestion made that it rests primarily on the minuteness of the original effective dose attained by needle inoculation.

Reason is shown for the suggestion that the virus of crinkle "A" is a mixture of viruses, and that one element at least is responsible for the crinkle-mosaic type, and another for the streak type of symptom.

Streak itself, however, is clearly not the clinical expression of but one specific virus. Thus President can convey to Great Scot a lethal streak which produces no streak symptoms on itself; on the other hand, President can be killed in a few days by grafting with certain relatively healthy-looking Uptodate or Duke of York plants.

The modified disease produced by inoculation in its further passage to the Potato produces only the modified disease. There is no increase of virulence.

Infection by means of imbibition of virus-infected juice has been effected, and resulted in a modification of clinical symptoms similar to that induced by needle inoculation.

The different reaction of *Datura Stramonium* plants to crinkle "A," as induced by grafting and needle inoculation respectively, is described. A typical crinkle "A" inoculation reaction is defined.

The reaction of Datura to interveinal-mosaic and simple mosaic is compared with that of crinkle "A." The reaction to para-crinkle is distinct and will be considered in a further communication.

Passage of the virus of crinkle "A" through Datura modifies its virulence, so that when transferred to a healthy Potato it produces mosaic instead of

crinkle, except in the case of Epicure and Arran Crest, where a lethal streak develops.

Notwithstanding the reduction of virulence induced in the Potato by passage through Datura, reinoculation to Datura always produces the same reaction as does the original crinkle, whether it be derived directly or indirectly from President or any other varieties here considered.

A series of " double grafts " between Potato and Datura showed that the modification of symptoms induced by Datura is dependent on the interaction of the virus and the metabolic product of the actively growing Datura leaf, and that passage of the virus through a solid section of Datura stem has no such effect.

The symptoms of crinkle " A " in the varieties Arran Banner, Arran Comrade, Champion and Irish Chieftain, are described, and the reaction of the crinkle " A " as found in them on other varieties and on Datura and Tobacco discussed. The crinkle of Irish Chieftain in particular is examined in considerable detail.

Tobacco plants infected by inoculation, as well as by grafting with crinkle " A " of President, react, but the reaction is very variable in degree. Passage to further Tobaccos was carried out to the third generation. No change in virulence was observed as regards the symptoms in the Tobacco itself.

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DESCRIPTION OF PLATES.

Plate 1.

Fig. 1.—*President.* Pot No. 250.—Infected with crinkle "A" in previous season. Dwarfed. Lower leaves harsh, not fully developed. Apical growth showing crinkle symptoms. Photographed early in the season.

Fig. 2.—*President.* Pot No. 249.—Leaf of plant infected by grafting with crinkle "A." Note the dwarfing, the deformity, waving and ruffling of the leaflets. The whole leaflet is twisted on its axis. The apex and the lateral edges curved downward. The mottling tends to be blotchy, begins from the veins and extends outwards. The veins are cleared.

Fig. 3.—*President.* Pot No. 250.—Sister plant to fig. 1, photographed late in the season. The characteristic symptoms have disappeared, the stems are barer.

Fig. 4.—*Arran Victory.* Pot No. 250 A3.—A leaf of a plant infected by grafting crinkle "A." Neither deformity, waving nor ruffling are the characteristic symptoms as in President. Mottling is bright, and clearly associated with the veins.

Plate 2.

Fig. 5.—*Arran Victory.* Pot No. 249 A.A2.—A leaf of a plant infected by grafting crinkle "A." Photographed late in the season. The leaflets are of a fair size and there is only a slight waving. The mottling is represented by large diffuse areas of paler green in which traces of the original brighter veinal markings are still visible. The surface is smooth and glazed.

Fig. 6.—*Arran Comrade.* Pot No. 249 A.Cde.—Infected by grafting crinkle "A." Clearing of veins is very well marked and with it a certain amount of mottling spreading from the veins. The waving of the leaflet edges is pronounced.

Fig. 7.—*Abundance.* Pot No. 249 Ab.—A leaf exhibiting a mild interveinal mosaic following grafting with crinkle "A."

Fig. 8.—*Epicure.* Pot No. 248 E.—Earliest symptoms following infection by grafting crinkle "A"; necrotic flecks on growing leaflets.

Fig. 9.—*King Edward.* Pot No. 250 K. Ed.—Necrotic flecks growing rapidly—Leaf drop and death ensuing later as a result of grafting crinkle "A."

Plate 3.

Fig. 10.—*King Edward.* Pot No. 499.—Final stage of infection by crinkle "A" in King Edward following grafting. Note the President-crinkle scion is relatively vigorous.

Fig. 11.—*Great Scot.* Pot No. 250 Gt. S. 3.—Infected by grafting with President crinkle "A"; the veins are cleared, necroses present; when the latter are not too advanced they are accompanied by some deformity of the leaflet.

Fig. 12.—*President.* Pot No. 234.—A leaf with mosaic mottling of crinkle-like type formed in relation to the veins, following infection by needle inoculation of crinkle "A."

Fig. 13.—*Arran Victory.* Pot No. 250 A. 2.—A leaf with an interveinal mottling following infection by needle inoculation of crinkle "A."

Fig. 14.—*Datura.* Pot No. 850, 4.—A leaf following infection by grafting with President crinkle. Dwarfing, some deformity, spotty and blotchy mottle.

Fig. 15.—*Datura.* Pot No. 250 Dat.—Leaf; the fine network mottle due essentially to the clearing of the entire vein system is seen spreading from the base.

Fig. 1.

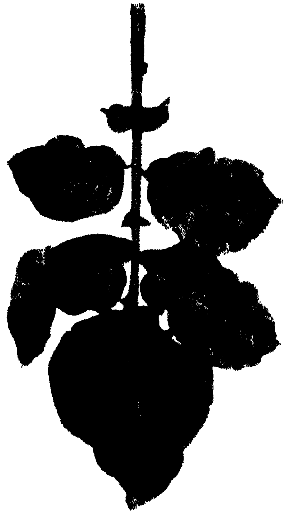


Fig. 2.

Fig. 3.

Fig. 4.

(*Facing p.* 82.)

Fig. 5.

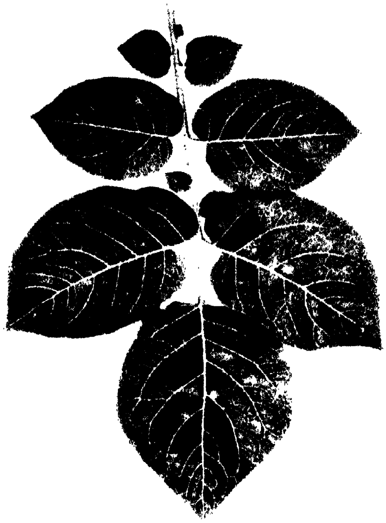


Fig. 7

Fig. 8.

Fig. 6.

Fig. 9.

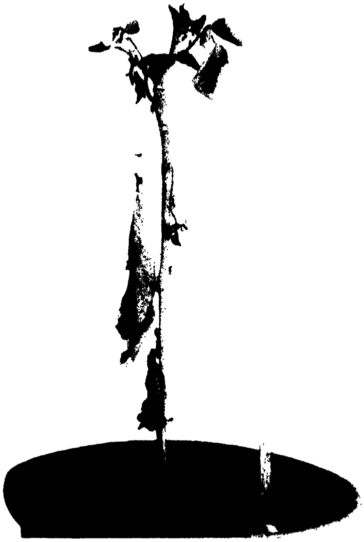


Fig. 10

Fig. 11

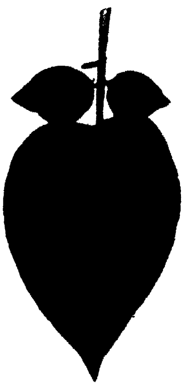


Fig. 12.

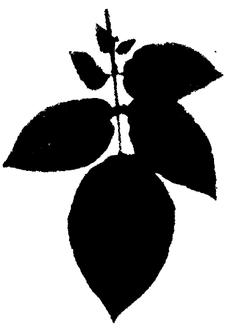


Fig. 13.

Fig. 14.

Fig. 15.

Fig. 16.

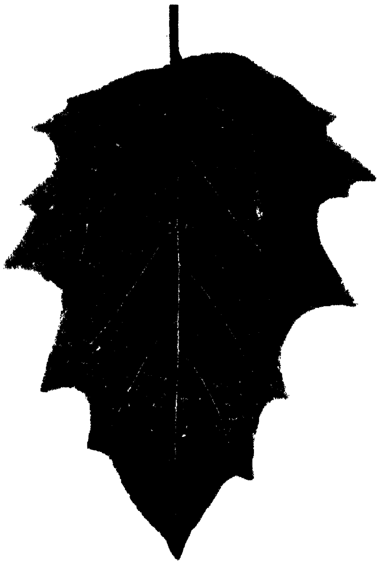


FIG. 17.

FIG. 18.

FIG 19.

FIG. 20.

FIG. 21.

FIG. 16.—*Datura.* Pot No. 250 A. 4, Dat. 1.—Leaf following inoculation of crinkle "A" from Arran Victory. Here the coarsest type of network mottle is seen with the pale areas bounded by the cleared veins with their dark green edgings, as well as the later interveinal type of mottling where these green borders have become bleached and the pale areas coalesced.

PLATE 4.

FIG. 17.—*Datura.* Pot No. 249 Ab. 1, Dat.—Modified reaction produced by inoculation with President crinkle "A" after its passage through Abundance. The dark borders to veins are much wider, the pale interveinal areas are faint and filled with fine dots which form small rings.

FIG. 18.—*Datura.* Pot No. 1025.—Leaf following inoculation with Arran Victory mosaic. The pale areas form first on either side of the main veins which for the most part are not bordered by dark green lines.

FIG. 19.—*Tobacco.* Pot No. 250 Tob. 2.—White Burley variety inoculated with President crinkle "A." Mottling and ring formation.

FIG. 20.—*Arran Banner.* Pot No. 245.—Suffering from crinkle "A."

FIG. 21.—*Irish Chieftain.* Pot No. 204.—Suffering from crinkle "A."

595·772—157

The Remarkable Adaptation by which a Dipterous Pupa (Tabanidæ) *is preserved from the Danger of Fissures in Drying Mud.*

By W. A. LAMBORN, Medical Entomologist, Nyasaland.

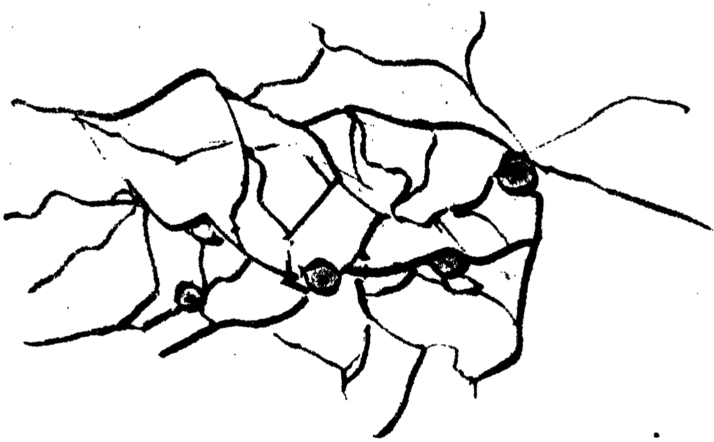
(Communicated by Prof. E. B. Poulton, F.R.S.—Received October 8, 1929.)

[PLATE 5.]

[The observations described in the following brief paper were recorded in a letter, written August 14, 1929, from Fort Johnston, Nyasaland, by my old friend the author. They are so remarkable and novel that it seemed only appropriate to extract the relevant passages and communicate them at once to the Royal Society. The specimens exhibited to the meeting and represented in the illustrations arrived shortly after the letter.—E.B.P.]

I have been trying to make a study of the Tabanidæ, particularly as to breeding-places and seasonal occurrence, with a view to ascertaining if I can, when I have learnt more about them and how to handle them, if they are concerned in the indirect communication of certain animal trypanosome infections. I had not hoped to add to our present knowledge any additional bionomic facts, for Dr. Neave has worked out the subject so very thoroughly

in this country. Nevertheless, fortune has favoured me and I think that I have discovered a feature in Tabanid life-history which is new to science. On August 10, 1929, while walking across a hollow which serves to collect



TEXT-FIG.—From the Author's Diagrammatic Sketch of Cracks and Tops of the Mud Cylinders.

water as a game.pool in the rains, I noticed at the intersection of cracks which ramify in every direction in the dry mud and are often several inches across and a foot or two deep at this season, a smooth disc of mud, about the size of a penny piece, slightly concave and perfectly circular, with the central area about the diameter of a lead pencil, crumbled away. On closer examination I found that this disc was the top of a cylinder of mud, and that I was able to remove it readily, thus finding it to be about 3½ inches in height. On cracking it I found a pupa which I recognised as that of a Tabanid. Having discovered one I had no difficulty in finding numbers and soon obtained a hundred and nine cylinders, forty-eight of which were tenanted by living pupæ. The rest were empty or contained only the pupal shells, often projecting from the top.

The cylinders really are wonderful structures. They must be made by the larva, and it is easy, I think, to make a fairly safe inference as to its procedure. From an examination of the exterior it is obvious that the larva tunnels through the mud when in a plastic state, moving spirally, round and round in an almost perfect circle, so that the mud intervening between the spiral turns is so reduced as to form a very thin partition wall. It probably begins on the surface—hence the concavity—at a time when the mud just begins to harden, and

gradually works down as the water dries out. Having reached the required depth the larva, as proved by the grooves left on the pillar, works its way upwards, cutting sharply across the close spiral, so as to rise quickly. Then, at about half-an-inch below the surface the larva burrows into the cylindrical mass it has formed, seals up the entrance and hollows out the interior to form the pupation chamber. The sealed hole is a feature common to all the cylinders that I have examined.

I surmise that the aperture prepared for the exit of the imago is the work of the pupa, for it is plugged with dry crumbled mud which, contrasting with the rest of the top, is clearly of later formation. This must be the main function of the armament at the head of the pupa, a feature common to such as have to make their way to the surface in preparation for emergence, for example, those of certain Aegeriid moths, or Bombiliid and Asilid flies.

The purposes served by these cylinders seem to me quite clear. They render the pupa absolutely safe from sudden exposure when general cracking of the surface of the mud takes place. The length of the chamber permits the pupa, as I have observed, to move up or down at will, whereby it is enabled to escape the effect of extreme heat at the surface during the midday hours, and to obtain a certain amount of moisture even at the present hot season. It also, doubtless, enables the pupa to escape the attacks of enemies.

You will, I am sure, agree when you see the cylinders that they are simply marvellous structures. I am now sending you the second one I found (the first having been broken to pieces).

I am greatly exercised in mind as to the species of Tabanid which will emerge. I have long known the mud-hole as the haunt of *T. biguttatus*, Wied., having seen females ovipositing there when the pool is full (though in Neave's experience the species favours sandy river beds for breeding). So far seven,* all of this species, have emerged. Should all the others turn out to be the same it will give me great joy to search for the pupal chambers of other species. The discovery will have some practical value, for it will afford a sure means of coping with the larvæ and pupæ, if need be, and still better it will enable the worker readily to secure many clean specimens for experimental work, it being laborious and unprofitable to dig for them in haphazard way, as I have previously been obliged to do. I ought now to be able to work out their parasites—always a fascinating pursuit.

By the way, I have been interested to see the two forms of the female, the

* Ten out so far: all *biguttatus*. August 18.

common variety, with white hair clothing the thorax, and the rarer one, with golden hair, very bright in the living insect. I have now seen this latter form for the first time outside a museum.

W. A. L.

In a later communication (November 16, 1929) Mr. Lamborn wrote—" The rim of the cylinders is flush with the general surface of the clay. The pupa is able to move up and down inside the central chamber. It probably remains at the bottom until shortly before emergence so as to escape the terrible heat, the bottom of each cylinder being damp and softer than the upper part, which is of the consistence of brick. So far I have only got pupæ from *two* situations, though I have found cylinders innumerable elsewhere, and the only Tabanids that have come out have been *T. biguttatus*."

[Major E. E. Austen, D.S.O., has informed me that he is confident that the observations recorded above are entirely new. At his kind suggestion the following memoirs were consulted, his opinion being confirmed :—

1. H. H. King, Report on Economical Entomology (' Rep. Wellcome Res. Lab., Gordon Mem. Coll.,' vol. 3, p. 212 (1908), " Life History of *Tabanus biguttatus*").

2. S. A. Neave, " The Tabanidæ of Southern Nyasaland, with notes on their life histories." (' Bull. Ent. Res.,' vol. 5, pt. 4, p. 287 (1915).)

3. Werner Marchand, " The Early Stages of Tabanidæ (Horse-flies)." (' Monographs of the Rockefeller Inst. for Med. Res.,' No. 13, Nov. 15, 1920. New York.)

H. H. King bred *Tabanus biguttatus* under artificial conditions, while Neave observed the larva in sandy soil unsuitable for the formation of cylinders. It will be of great interest to undertake specially directed experiments in order to determine whether this elaborate adaptation is called forth or withheld by appropriate or unsuitable conditions, respectively ; also to ascertain whether the cylinders formed by the larvæ lose their moisture at the same rate as others produced artificially and submitted so far as possible to the same conditions. Should the former retain their moisture longer than the latter some treatment of the surface by the larva may be suspected. The concave top of the cylinder may also serve to collect moisture from the surrounding surface.—E.B.P.]

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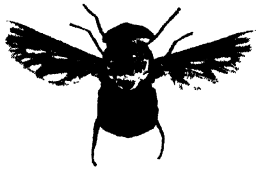

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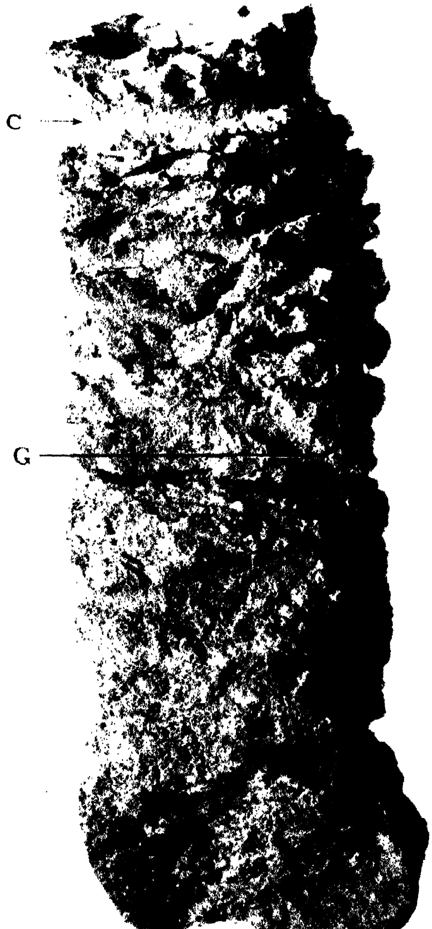

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Alfred Robinson photo.]

EXPLANATION OF PLATE 5.

(All the figures are of the natural size.)

Fig. 1.—The top of the mud cylinder represented in figs. 4 and 5. This view from above shows the concave surface with the central aperture for the emergence of the imago.

Fig. 2.—The pupal shell of *Tabanus biguttatus*, Wied. The size of the larva which cuts off a cylinder (similar to figs. 4 and 5) from the surrounding mud may be inferred from this figure, allowing for some considerable contraction at pupation.

Fig. 3.—The imaginal *T. biguttatus* which emerged from the pupal shell shown in fig. 2.

Fig. 4.—A mud cylinder seen from the side. A groove or collar below the top is indicated by C. The close spiral tunnel by which the larva has cut off the cylinder is shown by the nearly horizontal grooves on the face and their sections in the R. and L. contours. The tunnel made by the larva in its ascent, cutting across the close spiral of its descent, is shown at G. The cylinder was broken in transit but is otherwise perfect.

Fig. 5.—The same cylinder turned round to show the entrance E, sealed by the larva after it had penetrated the cylinder preparatory to constructing the central pupal chamber.

552 · 323 · 5 : 539 · 383

Ball-and-Socket Jointing in Basalt Prisms.

By F. W. Preston, Ph.D., F.R.G.S., F.I.P., A.M.I.C.E.

(Communicated by M. W. Travers, F.R.S.—Received November 4, 1929.)

[Plate 6.]

This paper is a brief explanation of this particular phenomenon, well developed in the Giants' Causeway and many other basalt sills, a phenomenon that has puzzled so many physicists and geologists that it is worth a little special comment, although the explanation is exceedingly simple and conforms to the same general principles governing the fracture of brittle solids as are set out in the writer's more general papers on the subject.* A good summary of our knowledge of prismatic structure in igneous rocks is given by R. B. Sosman in the 'Journal of Geology' for April, 1916.† References not tabulated in the footnotes of the present paper will be found listed by Sosman.

* See especially 'J. Soc. Glass. Tech.,' 1926, vol. 10, p. 234 ; also "Spalling of Bricks," 'J. Amer. Ceram. Soc.,' vol. 9, p. 654 (1926).

† Sosman, 'J. Geol.,' vol. 24, p. 215 (1916).

Columnar structure in basalt sills is caused by lateral contraction accompanied by vertical compression. Like all igneous jointing it is cellular in character and differs from ordinary jointing in the suppression of the tension in the vertical direction.* The question has been raised by French physicists whether prismatic structure may not arise in "tourbillons cellulaires" or convection prisms.† Sosman finds little evidence of it in the cases he has examined, and in the present paper I shall show that the evidence that appears to him most favourable to the convection-prism view, is in fact proof positive of the contractional view.

Basalt may split into columnar structure either from one face of the sill, or from both, or from points. In the latter case the prisms radiate and their diameters increase with distance from the point of origin. Let us suppose for the moment that we have a sill of uniform thickness, which has chosen to split into parallel vertical columns. The dead weight of the prisms themselves plus the dead weight of the overburden should, if the columns are strictly vertical, preserve them in compression, and there should be no need of cross-fracturing, were it not for secondary thermal stresses.

So long as the sill remains unruptured the isothermals are horizontal sheets, and, when the columns have formed, the outsides of each column are as hot as the inside. But now arises the possibility of the column losing heat to air or water in the cracks. Let us suppose that cold air or some other quenching medium gets access to the interstices. Then the column is chilled all around its outside while the interior is still hot. The outside tends to contract and cannot do so, since the whole column, though possibly red-hot, is solid, for it must be solid before the vertical prismatic cracks can form. The outside is therefore thrown into tension. If the quenching be severe enough, this tension may be sufficient to overcome the natural compression due to the overburden, so far as the outer layers of the prism are concerned, and to produce a net tension that exceeds the tensile strength of the basalt. A crack then forms at one side of the column, at a point such as A (figs. 1 and 3) and at right angles to the length of the column. It rapidly spreads around the column, its advance being indicated by the successive dotted lines *b*, *c*, *d*, *e*, *f*, in fig. 3. Finally the spot inside the circle *f* is left; this does not break at this stage, but remains free from appreciable thermal stresses and carries the compressive load due to the whole of the upper part of the column and its overburden. In fig. 2 is shown the temperature distribution across a vertical section of the column

* Preston, 'J. Soc. Glass. Tech.,' vol. 10, p. 263 (1928).

† Sosman, *loc. cit.*, p. 219.

at various stages. At A is the even temperature before the prismatic structure develops. At B is shown the effect of the sudden admission of a quenching

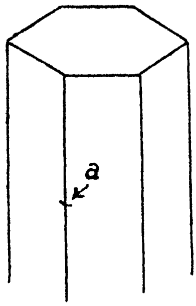

Fig. 1.

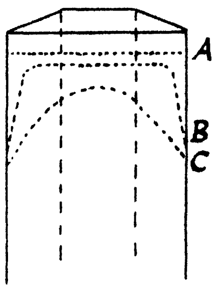

Fig. 2.

medium to the cracks. At C is the final temperature gradient, after the cooling medium has been admitted for some time.

Now the quenching medium must come from somewhere, above, below, or from one side, and it must in general be circulating or continually replaced. The vertical crack does not of itself constitute a cooling device; it needs some supply of "cold," which can only come from the circulating fluid medium. Let us suppose for a moment that this cooling agent comes from above, so that the upper part of the column is comparatively cold, down to the level of the opening fissure at *a*, fig. 4, while below *a* the column is somewhat warmer. Then if we cut the column through by a horizontal plane at the level of *a*, the upper part as a whole would shrink to the dotted lines, becoming smaller in diameter than the lower part. The tendency of the upper

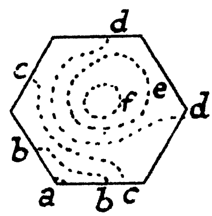

Fig. 3.

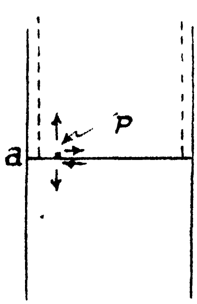

Fig. 4.

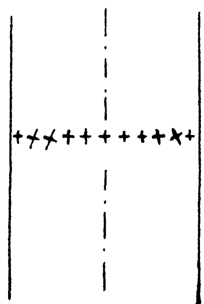

Fig. 5.

part to contract and of the lower to stay extended, exerts a shearing stress on the horizontal section, so that a particle of basalt at P, say, is being

H 2

stretched in a vertical direction and being sheared horizontally. This combination of shear and tension causes the maximum tension at the point to be inclined somewhat to the vertical, in accordance with well-known mechanical laws, so that the directions of the "principal" stresses are not vertical and horizontal throughout the cross-section, but have the angles shown in fig. 5. The principal tension is strictly vertical only at the edges and the centre of the column.

Now in a brittle substance like glass or basalt, a slowly-advancing fissure progresses continually at right angles to the principal tension. Since at the boundary between the rock and the circulating medium the tension is parallel to the face, the fissure that enters there must enter at right angles to the face. This results in the ball-and-socket joint having a square lip, on this side at least, and not being a simple basin. Once inside the boundary, however, the advancing fissure finds itself in a region where the principal tension, as shown in fig. 5, is slightly inclined to the vertical, and consequently the advancing crack bends downwards slightly from the horizontal (fig. 6). The downward tilt

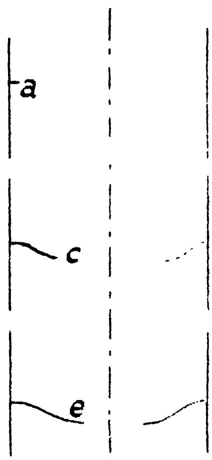

Fig. 6.

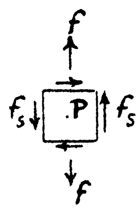

Fig. 7.

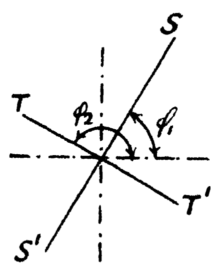

Fig. 8.

increases for only a short distance, and then, as the fissure approaches the central parts of the column, the principal stress tends to become vertical again, as we have seen; accordingly the fissure becomes more nearly horizontal in this region. This chain of circumstances gives the characteristic ball-and-socket or basin-like form to the cross-joint.

The fissure will not generally quite complete its journey. If it succeeded in

circumscribing the column before advancing deeply into the interior, it will leave a small circle of rock near the centre unfractured. To fracture this we need an earthquake or some such disturbance, or that the sea or human agency quarry the rock. The fracture is then completed, but the central spot is a newer fracture and of a different appearance. It is thus generally visible as a distinct core.* James Thomson thought this was the origin of the whole fracture, instead of the last of it.† Dauzère‡ thought it evidence in favour of his convection-cell theory, but Sosman§ proved, by polishing a section, that the core was purely superficial and did not extend beneath the surface.

I have explained a two-dimensional equivalent in glass,|| and an even closer three-dimensional analogue.¶ If the fissure advances deeply into the heart of the column, before it completely circumscribes it, the last part to remain unfractured will be at one edge. The fissure, on completing this part of the fracture, will then tend to become tangential to the exit face. This requirement, that the fissure make its exit tangentially, is due to the same mechanical laws that require it to enter perpendicularly. At the edge the principal stresses must be parallel to and perpendicular to the face. Before a fissure forms the greatest of these is the parallel stress; when the fissure has cut the column into two, except for a small zone near one edge, the greatest tension becomes the perpendicular one, and accordingly the fissure bends away and becomes nearly tangential. I have explained this in more detail in the other papers elsewhere cited.

The photographs reproduced on Plate 6 are by Mr. R. J. Welch of Belfast. The upper shows particularly well a number of square-lipped basins, one corner at least being square-lipped and showing the point of entry of the cross-fracture. Some such lips are marked by the letter A. The lower photograph shows very clearly a number of corners where the fissure made its *exit*, the arrows marked B pointing to some of the clearest of them. It will be observed that the concavity of the fracture sometimes faces upwards and sometimes downwards—sometimes it is a basin and sometimes a dome. This seems satisfactory evidence that the chilling of the columns was somewhat "spotty," the circulating medium apparently coming sometimes from above

* Sosman, *loc. cit.*, pp. 227–229.

† 'Belfast Nat. Field Club. Ann. Rep.,' vol. 7, pp. 28–34 (1869).

‡ 'J. Physique,' vol. 6, pp. 892–99 (1907); vol. 7, pp. 930–34 (1908); 'Assn. Franç. Av. Sci.,' pp. 289–96, 436–38 (1908).

§ Sosman, *loc. cit.*, p. 228.

|| 'J. Soc. Glass. Tech.,' *loc. cit.*, p. 245.

¶ 'J. Soc. Glass. Tech.,' *loc. cit.*, fig. 23.

and sometimes from below. The concavity in each case should form the end of that part of the column that was the warmer when the cross-fracture formed.

Our theory requires that the basins should all be of a somewhat shallow contour, except of course at exit edges. For if at the point P in fig. 4, the vertical tension was f and the shear stress on the horizontal plane was f_s (fig. 7) while the fracture was forming, then in accordance with the laws of physics the direction of the principal planes will be given by $2\phi = \tan^{-1}(-2f_s/f)$ (fig. 8) so that, since f and f_s are both positive, ϕ_1 lies between 45° and 90°, while ϕ_2 lies between 135° and 180°. Hence the fissure, which will dip down at the angle TT′, will never dip at angles greater than 45° to the horizontal, and generally it will be much less. Hence the depth of the deepest bowl can hardly exceed one-fifth the diameter of the column, unless the advancing fissure radically redistributes the stresses. This ratio of one-fifth follows from the fact that a spherical bowl whose lip is at 45° to the horizontal has a depth of $(\sqrt{2} - 1)/2$ times its lip diameter, or approximately one-fifth. Of course, in the case of fissures making their exit from a face, no such calculations are permissible.

The resemblance of the cross-fracturings of basalt columns to the fractures of a spalled silica brick, broken by heating in a furnace and then quenching in water, is very close. The brick, of course, is rectangular in cross-section, while a basalt prism is usually more or less pentagonal or hexagonal, but in each case we are apt to find a square entry at one corner, where the fracture began, a basin-like hollow in the centre, caused by the combination of shear with tension, a tangential exit on the last corner to give way, and one or more corners, which are partial exit corners, but not actually the last exits, with an angle of some intermediate value.

Thus both the original prismatic structure of basalt sills and the subsequent cross-fracturings of the ball-and-socket type, owe their characters to the simple requirements of brittle bodies undergoing fracture. In an isotropic solid such as glass, or for the present purpose basalt, the requirements always are—

(1) Failure occurs in tension.

(2) The fissure advances at right angles to the maximum tension at its head.

If a prismatic structure is to develop we need the additional requirement—

(3) The tension should be equal in two directions, usually the two horizontal directions, while in the third direction, usually the vertical direction, the tension should be small or replaced by a compression.


A
B
B


B

And for the formation of ball-and-socket jointing we need also—

(4) After the prisms have formed, they must be quenched by a cooling medium percolating through the prismatic cracks.

I would like to thank Mr. Welch for permission to use his photographs; Prof. J. Kaye Charlesworth, of Queen's University, Belfast, Ireland; Mr. W. B. Wright, of the Geological Survey of England; Dr. E. E. Lowe, Director of the Leicester Museum, England; and Dr. R. B. Sosman, till recently of the Geophysical Laboratory, Washington, for information on a variety of points that has proved very serviceable.

581.12.036:634.11

Physiology of Fruit.—I. Changes in the Respiratory Activity of Apples during their Senescence at Different Temperatures.

By Franklin Kidd and Cyril West, Food Investigation Board of the Department of Scientific and Industrial Research, and Low Temperature Station for Research in Biochemistry and Biophysics, Cambridge.

(Communicated by Dr. F. F. Blackman, F.R.S.—Received November 19, 1929.)

CONTENTS.

Introduction.

For some years there has been in progress, at the Low Temperature Research Station at Cambridge, a series of researches dealing with the influence of important variables upon the metabolism of plant tissues and organs. These variables are race, age, nutrition and temperature. While these researches are academic in character, they have been planned to explore a field of enquiry in which an advance in our knowledge of general principles appears likely to assist industrial progress. The economic background in the present instance is to be found in modern necessities for the long storage and transport of living plant tissues—the fruits and vegetables of commerce—for consumption as food.

In pursuance of this general plan attention has been concentrated on the apple, and, by the present authors, on the study of its respiratory metabolism. In the first place studies of the gaseous exchange in respiration only were made. In later researches, in collaboration with the Hon. Mrs. Onslow, the investigations on gaseous exchange have been linked up with an investigation on the concomitant changes in metabolites.

The data of certain of these researches have been set out in Annual Reports of the Food Investigation Board from 1922 onwards. The present communications are designed to bring out the main scientific problems that have been attacked. The problem dealt with in this paper is indicated in the title.

The temperatures selected for study were chosen arbitrarily. They were constant temperatures of 2·5° C., 10° C., and 22·5° C., and the uncontrolled temperature of the laboratory, averaging 15° C. A sample of graded Bramley's Seedling apples was gathered from a selected orchard on October 1, 1920, and several hundreds of perfect specimens were weighed on the second day after gathering, and stored at each of the temperatures stated above in single layers on wooden trays.* Simultaneously, the recording of CO_2-production at the three constant temperatures 2·5°, 10°, and 22·5° C. was commenced.

Preliminary Considerations on Life Duration and Sampling.

It would be ideal if it were possible to select individual apples identical in their physiological condition for the purpose of the comparisons of temperature in which we are interested in this research. Identity in physiological condition is not attainable. It was therefore necessary to use as the experimental

* The apples used by Blackman and Parija (2) for the investigations of which an account has already been published, were specimens from the above sample stored at 2·5° C.

units samples large enough to ensure that the results obtained represented within narrow limits the average behaviour of the population at the temperatures studied. Samples of 4, 5, or 6 apples were used, the behaviour of which may be regarded as representing, within limits of 10 per cent., the average behaviour of the population as a whole. On two occasions a duplicate sample was employed to check this point. The results agreed to within 3 per cent. of each other. At all the temperatures employed in this research the apples eventually died as the result of fungal disease.

A difficulty which arises when samples of apples are used for following changes in respiratory activity with time is that there comes a time when one will begin to rot. The CO_2-output of a diseased apple rises rapidly with the progress of the invasion of the fungus and the concomitant disturbance of the tissue. Whenever, therefore, any apple showed signs of fungal disease it was removed, and the record of respiratory activity continued with the remaining sound apples. When more than half the sample had become affected by disease, new apples were added or a completely new sample taken. The records of respiratory activity thus tend in the later stages of senescence to be based upon the longer-living variants of the population.

In order to compare the total carbon dioxide outputs during senescence at different temperatures, we must consider the question of how to determine the points in time on the records of respiratory activity which correspond to the average duration of life at the various temperatures.

The individual variation in length of life in a population of apples held at any constant temperature is more pronounced than the individual variation in respiratory activity. In a population of Bramley's Seedling apples, studied subsequently (1926) by the authors, it was found that the duration of life at 12° C. varied from 75 to 317 days, with a mean value of 163 days (3). The chances were between two and three to one *against* any individual apple chosen at random having a life duration within $\pm$ 10 per cent. of that of the average. On the other hand it was found that three out of every four apples showed a respiratory activity lying within $\pm$ 10 per cent. of the average, the extreme being approximately $\pm$ 30 per cent. of the average.

Samples of four to six apples are, therefore, clearly too small to give a reliable estimate of the average duration of life of the population.

The average duration of life at 2·5° C. could, however, be determined approximately from descriptive records of the condition of the main stock of apples made from time to time, and a period of 205 days was indicated. The duration of life at the other temperatures was then calculated from the relationship

between length of storage life and storage temperature which we have elsewhere (4) established from the results of several studies of life duration conducted on the scale of 100 and more apples to a sample. Values of 41·5 days at 22·5° C. and 97 days at 10° C. were obtained in this way.

Experimental Results.

The records of respiratory activity at the three temperatures from the time of gathering onwards are presented in fig. 1, and the details of the experiments

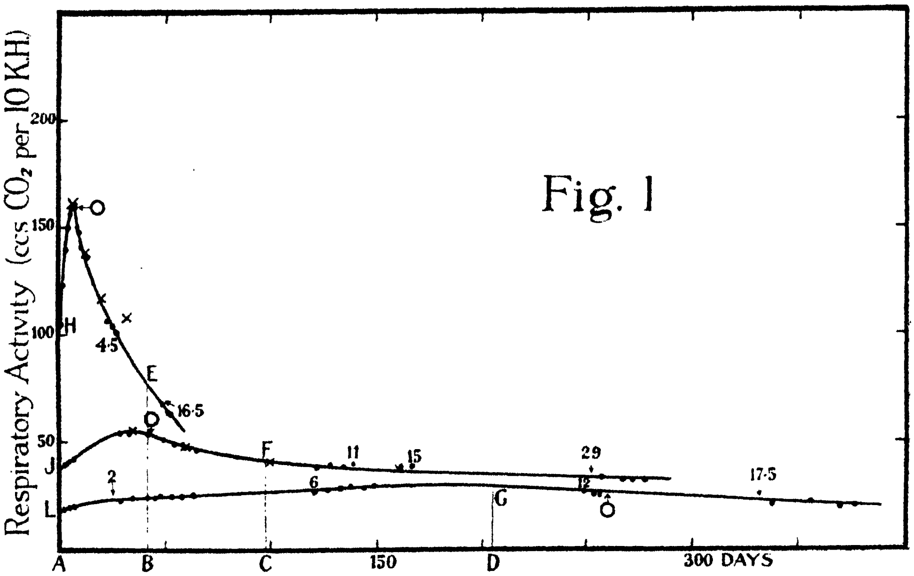

Fig. 1.—Respiratory activity of Bramley's Seedling Apples at 22·5° C. (top curve), at 10° C. (middle curve), and at 2·5° C. (bottom curve).

are given in Tables I, II and III of the Appendix. The heavy curves represent the progress of the change in the respiratory activity which accompanies the advance of senescence. The dots on these curves are the observations made. The thin vertical lines EB, FC and GD mark the points in time corresponding to average duration of life. The arrows adjacent to the curves indicate the points at which the samples under observation were replaced by fresh samples of apples from the stock of apples stored at each temperature. The Arabic figures indicate the percentage loss of fresh weight since gathering of the fruit under observation.

Fig. 2 sets out the average loss of fresh weight in the apples stored at each temperature, the vertical lines marking the duration of life in each case. The

loss of weight by evaporation from the fruit was more rapid in open storage than in the respiratory containers.

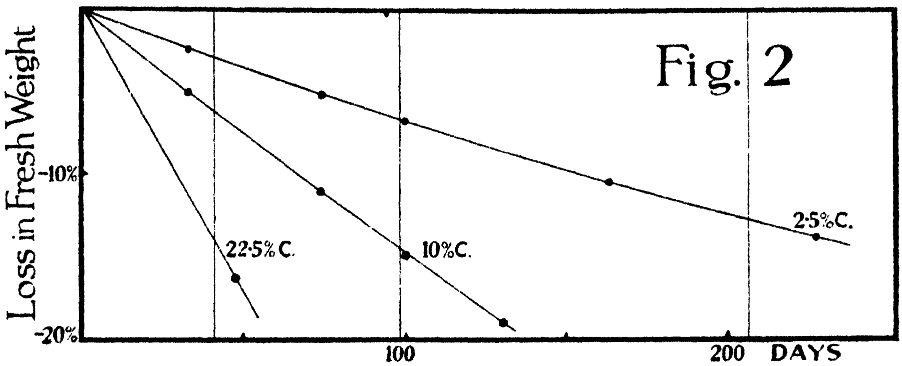

FIG. 2.—Loss in fresh weight of Bramley's Seedling Apples stored at 22·5° C., 10° C., and 2·5° C. respectively. The average duration of life at each temperature is marked by a vertical line.

The records of respiratory activity indicate a set of facts of considerable interest. In the first place the progress of senescence is characterised at all temperatures by a rise, followed by a fall, of respiratory activity. Secondly, the "peak"* value of respiratory activity at all the temperatures is about 1·5 times the initial value. Thirdly, the time taken to reach the "peak" value varies inversely with temperature to a striking degree. Lastly, there is no constant relation in time between the onset of fungal disease and the occurrence of the "peak" value of respiratory activity at different temperatures. At the lowest temperature the onset of disease is practically synchronous with the peak, whereas at the highest temperature the duration of life is six times longer than the time taken to reach maximum respiratory activity.

Before discussing the significance of these facts it is convenient to deal with the question of temperature coefficients and with the question of the relation between total carbon dioxide production and duration of life at the three temperatures studied.

Temperature Coefficients.

(*a*) *Of Rate of Carbon Dioxide Production.*—The rate of CO_2-production is probably in the main controlled by the following factors—(1) effective concentration of substrate; (2) effective concentration of enzymes; and (3)

* The present writers have elsewhere (1) used the term "climacteric" to denote this stage in the senescent phase of the apple fruit.

temperature. Many factors both external and internal must influence both the effective substrate and the effective enzyme concentrations.

In order to ascertain the effect of temperature we must consider points on our records at which other factors likely to affect rate are presumably the same. The values obtained immediately after the simultaneous starting of the experiments at the three temperatures are no doubt the best points to take. The values are in the following ratio :—

2·5° C.		10° C.		22·5° C.
1·0	:	2·1	:	5·6

The values at the "peak" points of respiratory activity, viz., 30, 55, and 161 c.c. CO_2 per 10 K.H., show sensibly the same relation, *i.e.* :—

2·5° C.		10° C.		22·5° C.
1·0	:	1·8	:	5·4

These ratios agree substantially with those found by Gore (5), who compared the rate of evolution of carbon dioxide by apples at different temperatures for a short period immediately after gathering.

(*b*) *Of Absolute Rates of Acceleration and Deceleration of Respiratory Activity.*—As regards the effect of temperature upon the rate of change-in-rate of CO_2-production with advancing senescence, the following approximate ratio values are obtained from tangents to the slopes of the curves. These tangents are taken at the points of origin at zero time for the rise, and at the points marked with a circle and arrow for the fall.

	2·5° C.		10° C.		22·5° C.
Absolute rates of acceleration	1	:	9·0	:	122
Absolute rates of deceleration	1	:	8·4	:	113

(*c*) *Of Relative Rates of Acceleration and Deceleration of Respiratory Activity.*—The rate of CO_2-production has been regarded as a function not only of temperature, but also of the condition of the tissues in respect to effective substrate and effective enzyme concentration. Hence the *relative* rates of acceleration and deceleration should provide evidence as to the rate of senescent change in the condition of the tissues and of the effect of temperature upon this. Ratios for *relative* rate of acceleration and deceleration at the different temperatures are obtained by dividing the ratio figures for absolute acceleration and deceleration by the ratio figures for simple rate. This gives the following :—

	2·5° C.		10° C.		22·5° C.
Relative rates of acceleration	1	:	4·3	:	21·8
Relative rates of deceleration	1	:	4·0	:	20·2

The rates of senescent change can also be roughly judged and compared on the basis of the times taken to reach the " peak " value in the progress curves of respiratory activity. These times are 7 days at 22·5° C., 35 days at 10° C., and 190 days at 2·5° C. The ratios of the inverses of these times are :—

2·5° C.		10° C.		22·5° C.
1·0	:	5·5	:	27·5

It appears from the above that the influence of temperature upon the rate of change in the senescent drift of respiratory activity is markedly greater than its influence upon the rate of CO_2-production at any given stage in this drift.

An obvious corollary to the state of affairs set forth above is that at lower temperatures an apple liberates more CO_2 in the time taken for it to reach the " peak " value of respiratory activity than it does at higher temperatures. The actual amounts of CO_2 liberated at each temperature during the period from gathering to the " peak " were, per kilo. of original weight or apple gathered, as follows :—

At 2·5° C.	23·4 grs. CO_2
,, 10° C.	8·1 ,,
,, 22·5° C.	4·7 ,,

Total Production of Carbon Dioxide and Duration of Life.

The relation between respiratory activity and duration of life is next to be considered. The durations of life at the temperatures used, 2·5° C., 10° C., and 22·5° C. were as 205 : 97 : 41 respectively. The inverse of these figures are as 1 : 2·1 : 5·1.

The temperature coefficient of duration of life, when life is terminated by fungal attack, is thus of the same order as that of the rate of respiration, and is not of the order of the temperature coefficient of the rate or those changes in the respiratory mechanism which are responsible for the rise and fall of respiratory activity.

In accordance with the above it is found that the total amount of CO_2 liberated, and of dry matter lost from the time of gathering to the time of death, are approximately the same, no matter at what temperature the fruit has been stored and by how much its duration of life has been thereby extended or curtailed. The records enable us to determine these amounts by taking the areas enclosed within the zero ordinates, the progress curves of respiratory activity, and the vertical lines marking the termination of average life for each

temperature. These areas are lettered on fig. 1, AHEB, AJFC, ALGD, respectively for the three temperatures 22·5° C., 10° C., and 2·5° C. The values thus obtained are as follows:—

At 2·5° C.	12·84 litres CO_2
" 10·0° C.	11·35 "
" 22·5° C.	11·42 "

Thus the total amount of carbon dioxide liberated between gathering and death by fungal disease is approximately the same at any temperature and corresponds to about 1·6 grams of dry matter per kilo. of fruit.

The sugar and acid content of the fruit, when gathered, was between 8 per cent. and 10 per cent. of the fresh weight. Thus only between 16 per cent. and 20 per cent. of the apparent reserves of carbon hydrate had been exhausted at the time of death by fungal disease.

Consideration of the Factors responsible for Acceleration and Deceleration of Respiratory Activity in Senescent Tissues.

(*a*) *Effective Concentration of Substrates.*—In considering the upward and downward drifts of respiratory activity which accompany senescence and which occur at all temperatures, the question arises as to whether these changes in rate of CO_2-production are to be accounted for by changes in effective substrate concentration or in effective amount of enzymes.

Progressive changes in the concentration of the soluble metabolites, certain of which must form, directly or indirectly, the substrate of the immediate reactions concerned in the production of carbon dioxide, do proceed with senescence after gathering. Starch, if there is any present in the apple when gathered, disappears, and hexose sugars accumulate. The concentration of cane sugar, which is always present when the fruit is gathered, progressively decreases, as does also the concentration of acid. Soluble pectins at first increase and then decrease in amount, the increase taking place at the expense of insoluble pectin, and the subsequent decrease being due to further hydrolysis.

In the following table is shown the original composition of a representative orchard sample of Bramley's Seedling apples. Comparable samples were weighed, stored at a constant temperature of 1° C., and analysed at intervals. The absolute losses or gains occurring in the different important constituents are shown and serve to establish the point made above.

Initial composition of 100 grams of tissue.	Sucrose 3·47 gms.	Hexose 5·91 gms.	Total sugar 9·38 gms.	Acid 1·12 gms.	Total sugar and acid 10·50 gms.	Residue 3·21 gms.
Intervals —						
54 days	−0·86	0·57	−0·29	−0·10	0·39	−0·02
33 „	−0·38	0·11	−0·27	−0·12	−0·39	0·29
31 „	−0·10	0·20	0·10	−0·03	0·07	−0·24
27 „	−0·35	−0·05	−0·40	0·09	−0·49	0·04
61 „	−0·29	−0·08	−0·37	−0·15	0·52	0·08
44 „	−0·17	0·16	−0·01	−0·07	−0·08	−0·20
250 days	−2·15	+0·91	−1·24	−0·56	−1·80	−0·05

The only soluble substances present in the apple at the time of gathering in sufficient amount to provide the source of the carbon dioxide liberated during senescence are acid, cane sugar, and hexose sugar. Of these, hexose sugars, including the possibility of active forms of sugar derived during the inversion of cane sugar, are the only metabolites which increase in gross concentration. Evans (6) has shown that the hexose sugar of mature Bramley's Seedling apples is a mixture of fructose and glucose in the proportion of about 3·2 to 1. This proportionality changes slightly with advancing age, but in considering possible relationships between concentration and respiratory activity it will suffice here to argue from the total hexose values.

Available evidence from the work of Gore (5) and Haynes and Archbold (7), together with unpublished work of the present authors in collaboration with Onslow, indicates that the temperature relations of the rate of change in hexose accumulation and in sucrose disappearance are of the order of magnitude of 1 : 2 : 5, for the temperatues 2·5° C., 10° C., and 25° C., respectively. Let us suppose that the rise in respiratory activity, lasting in our experiments for 7 days at 22·5° C. and 190 days at 2·5° C., reaches a maximum at 2·5° C. at about the same time as the concentration of hexose sugars. Maximum respiratory activity at the higher temperature will then occur much earlier than the maximum hexose concentration, owing to the difference between the effect of temperature upon the rate of change in concentration of hexose and upon the rate of change in respiratory activity. It may be stated here also, that the percentage rise in respiratory activity—50 per cent. in the case of the present experiments, and as high as 100 per cent. in others not here reported—is greater than the percentage rise in hexose concentration in any case we have observed. It does not, therefore, appear that the rise in respiratory activity can be explained simply as being due to the rise in the concentration of hexose sugars in the tissues.

(*b*) *Effective Conentration of Enzyme.*—We can now return to consider further the phenomenon of the very large temperature coefficient of the rise in respiratory activity which accompanies advancing senescence.

Temperature coefficients of a high order of magnitude have been observed in connection with certain phenomena associated with changes in colloidal state, such as the denaturation of proteins, the inactivation of enzymes, and the life-duration of seeds. It is probable, therefore, that the acceleration and deceleration of respiratory activity observed during senescence is principally due to changes in colloidal state of the protoplasm.

The present authors have previously drawn attention to the lack of evidence that the rise in respiratory activity at the beginning of the senescent phase in the life-history of the apple is associated with any corresponding change in the concentration of any of the estimated constituents of the cells, and have tentatively put forward the hypothesis that the rise is due to "a change of state" in the colloidal matrix of the protoplasm (8). Under this head two possible types of change may be reserved for future consideration: (1) changes leading to a greater amount of effective enzymes; (2) changes leading to a greater effective concentration of substrate, either by elution or by increase in permeability of the surface of the protoplasm bordering the cell vacuole.

The Theories of Blackman and Parija.

It is impossible to leave the question as to the factors responsible for the rise and fall in respiratory activity with advancing senescence, without reference to the views of Blackman and Parija (2). These authors, as previously stated, worked with the same material as was used in this investigation. They took single apples at intervals from those stored at 2·5° C., and followed their respiratory history after removing them to 22·5° C. From the initial values obtained they deduced the general fact which we had previously established, that the progress of senescence is marked by a rise and fall in respiratory activity. They believe that a special phenomenon appears at the senescent phase which they entitle "a lowering of the organisation resistance." Organisation resistance expresses an "aspect of protoplasmic control of metabolic rate." Hindrance to reaction is pictured by them as being due to spatial separation of reactants by impermeable protoplasmic membranes, or to absorption or combination of reactants by stabilised components of the protoplasm. So far, their view, though differently expressed, agrees with ours in providing a theory to account for the rise in rate of CO_2-production as being due to a protoplasmic factor.

Blackman and Parija, however, go further. They narrow the general term "organisation resistance" to "hydrolysis resistance," and picture the rise in respiratory activity as being due to "an increased rate of production of effective substrate for respiration." They also believe that the fall in respiratory activity which succeeds the rise is due to "starvation," and that "starvation" relative to the change in organisation resistance proceeds much faster at higher temperatures. They state that "while the low rates of respiration that occur at 2° C. can be maintained, the higher ones proper to 22° C. cannot be maintained, but tend to fall off by what may be termed "starvation."

The present authors, while finding a certain amount of evidence in favour of the theory that the senescent rise in respiratory activity is conditioned by changes in the protoplasmic factor, do not find any evidence for a specific theory that the rise is due to a fall in "hydrolysis resistance" and to a related rise in effective concentration of respiratory substrates. As regards the assumption of Blackman and Parija that the "starvation" factor has a pronounced effect upon the form of the respiration-time curves at 22·5° C., but a negligible effect at 2·5° C., our observation that the consumption of carbon dioxide in respiration up to the time of the peak is five times greater at 2·5° C. than at 22·5° C. seems to present an obstacle to its acceptance.

Comparison with Leaves.

The course of respiratory activity of leaves after gathering has been studied by several workers (9 and 10). Blackman (9) was the first to observe that Cherry laurel and Tropæolum leaves during their senescence and "starvation," as isolated organs after gathering, appear to pass through two major phases of change. In the first phase respiratory activity falls off, steadily and more and more slowly as time proceeds, to low values. This first phase may be preceded by a short preliminary period of rising or level respiratory activity, or it may set in at once. The second phase is characterised by an increase in the rate of respiration which rises to a peak value, then again falls off. This second phase appears to correspond more or less with the yellowing of the leaf. Death with browning, fungal invasion, and a rise in the rate of carbon dioxide evolution, finally intervenes.

The respiratory behaviour of leaves varies also with their age at the time of their separation from the tree. Godwin and Bishop (10) state with regard to Cherry laurel that "young leaves (1 to 2 months from unfolding) show no hump and do not yellow. Browning and general disintegration commence at the same time as the final rise in the respiration. With leaves about 3 months

old, the first signs of a hump can be detected, and the commencement of yellowing is contemporaneous with this. The yellowing is overtaken by the final browning, and the corresponding final increase in the respiration. With increasing age from unfolding, a corresponding increase in size of the respiration hump is found, and in leaves 20 months old the peak rises as high as the initial value from which the respiration first falls. The onset of browning and fungus development and the final rise in respiration are correspondingly delayed."

With this picture of the drift of respiratory activity of leaves during senescence and "starvation" in mind, it is interesting to enquire whether the rise and fall in respiratory activity, which we have shown above to characterise the progress of senescence in the apple, is essentially the same phenomenon as the "hump" in respiratory activity which accompanies yellowing in the leaf after gathering.

In subsequent papers in this series it is hoped to publish results which indicate that the senescent rise in respiratory activity can occur while the apple is still attached to the tree. We have already given evidence showing that the phenomenon of the rise in respiratory activity of the apple does not always set in immediately after gathering, as it did in the case of the material studied in the present research (7). It is, in fact, a phenomenon which is not associated causally with separation from the tree. It may be initiated some considerable time after separation or it may occur on the tree before gathering. It appears thus to be inherent in the senescent drift of metabolism in the apple. As to whether the same can be said with regard to the leaf appears still to be a moot question. In experiments with cut leaves, the phenomena of their senescence as isolated organs in the dark may be complicated by concurrent phenomena of "starvation."

It has recently been stated by Hover and Gustafson (11) that "as leaves of corn, sorghum, wheat, and oats increase in age there is a decrease in rate of respiration (per unit dry weight), but that as the leaves become still older (past about middle age) the rate gradually increases." This statement is based on the evidence obtained from determining the initial respiratory activity, after a simultaneous gathering, of each leaf along a stem axis, from the oldest at the base to the youngest, or last-formed, at the tip. It is also shown that the first-formed leaves of the shoot, *i.e.*, the leaves which ultimately figure as the oldest in the stem series, start with a higher respiratory pitch than do later formed leaves, thus confirming previous work with leaves of *Helianthus annuus* (12). From the data presented, therefore, there is no

critical evidence that the respiratory activity of any individual leaf ever rises after middle age.

While, therefore, it is at present uncertain whether the senescent rise in the respiratory activity of the apple is a general characteristic of a definite phase in senescence in plant tissues, it is interesting to find that a similar phenomenon in starving leaves has been attributed to a change of state in the protoplasm.

Godwin and Bishop (*loc. cit.*) studied concomitantly the course of respiratory activity, the destruction of cyanogenetic glucoside and the yellowing of Cherry laurel leaves cut from the tree at various stages in the course of their growth. They found that the progress curve of disappearance of glucoside is sigmoidal, and that the corresponding modal curve of variation in the rate of change with time corresponds with the rise and fall in respiratory activity which characterises the yellowing stage in the senescence of these leaves. They were unable, however, to explain the rise and fall in respiratory activity as being due to an increased supply of respiratory material or to a change in sugar concentration, arising from the parallel phenomenon of glucoside disappearance, and concluded tentatively that both are due to an underlying change of state in the protoplasm.

Comparison with Other Fruits.

In a recent paper Gustafson (13) has shown that in the case of tomato fruits during growth there is a decrease with age in the rate of CO_2-production per unit weight of tissue; that a point of minimum production is reached about the time increase in size stops; and that this is succeeded by an increase in rate of CO_2-production which reaches its maximum when the fruits are orange to red in colour. From thence onwards the rate falls off continuously. Gustafson suggests that the increase in rate is due to a lowering of the H-ion concentration of the cell sap.

Summary.

1. The respiratory history of an apple during its senescence is characterised by a rise followed by a fall in activity.

2. The senescent rise in respiratory activity is attributed to a change of state in the protoplasm.

3. The change of state has a high temperature coefficient.

4. At all temperatures, death by fungal disease intervenes after approximately the same total amount of carbon dioxide has been evolved.

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APPENDIX.

Tabulated Data.

Note—(1) Respiration is expressed in cubic centimetres of carbon dioxide evolved, and of oxygen consumed per 10 hours per kilogram of fruit as gathered, *i.e.*, per 10 K.H. original fresh weight. Samples of from 4 to 6 apples were used.

(2) The apples (Bramley's Seedling) employed in these experiments were supplied as a uniform high-grade orchard sample, from a plantation on an outcrop of marl near Cambridge. Only apples of average size were used in the experiments.

(3) The measurement of carbon dioxide was made by the Reiset tower-absorption method using NaOH and the double titration with phenolphthalein and methyl orange. The volume of the desiccators used as apple containers was approximately 400 c.c. Moist CO_2-free air was drawn through the containers at a rate of approximately 40 litres per hour. Where the records show the occurrence of a long interval between observations the apples were removed from their containers. Wilting of the fruit was more rapid in open storage than in the respiration chambers.

Table 1.—Respiratory Activity in Air at 22·5° C. of Bramley's Seedling Apples from the second day after gathering onwards.

Time from starting experiment (days).	Observation periods (hours).	Respiration. CO_2.	Respiration. O_2.	Loss in fresh weight (per cent.).	Remarks.
1	18	105	101		(1) (2)
2	25	123			
3	26	140			
4	24	150			
5	24	156			
6	18	159	148		
7	20	161			
8	32	150			
9	26	148			
10	24	141			
13	48	136			(3)
		Interval of 9 days			
23	27	106			
25	46	104			
27	50	101		4·5	(4)
		Interval of 21 days.			
49	21	68		16·5	(5)
51	50	64			
52	23	63			(6)

(1) 8½ hours allowed to reach temperature equilibrium in respiration chamber.

(2) Four apples in sample used, weighing 613 grams.

(3) All apples sound.

(4) One apple showing a brown spot of fungal disease. Fruit yellow.

(5) A fresh sample of four apples used, weighing originally 578 grams. Fruit now yellow and wilted. These apples had been stored in an open vessel in the constant-temperature room at 22·5° C. and had thus lost much more water by evaporation than those enclosed in the respiration apparatus.

(6) All apples sound.

Table II.—Respiratory Activity in Air at 10° C. of Bramley's Seedling Apples from the second day after gathering onwards.

Time from starting experiment (days).	Observation periods (hours).	Respiration. CO_2.	Loss in fresh weight (per cent.).	Remarks.
2	42·7	40·0		(1)
4	47·4	41·4		
6	43·9	42·0		(2)
		Interval of 19 days		
28	77·0	54·7		
33	112·6	54·0		
41	199·0	53·8		(3)
		Interval of 5 days		
49	70·5	51·0		
54	116·3	49·0		
59	124·4	48·4		
65	145·6	46·3		
70	142·5	48·4		
		Interval of 44 days.		(4)
122	192·0	38·0	11·5	
128	146·2	39·5		
134	140·7	38·2		
139	129·3	39·7		
145	138·3	40·8		
150	116·7	41·0		(5)
		Interval of 6 days		
162	148·2	38·2	15	(6)
167	119·5	38·5		
		Interval of 85 days		
256	100·7	33·4	29	(7)
260	92·4	32·5		
266	144·0	31·2		
271	120·0	31·5		
277	147·0	31·5		(8)

(1) Six apples in sample used, weighing 824 grams.
(2) and (3) All sound.
(4) One apple commencing to rot and removed after interval.
(5) Two more apples commencing to rot and removed.
(6) Three remaining apples sound.
(7) A fresh sample of five apples used, weighing originally 860 grams.
(8) All sound.

Table III.—Respiratory Activity in Air at 2·5° C. of Bramley's Seedling Apples from the second day after gathering onwards.

Time from starting experiment (days).	Observation periods (hours).	Respiration CO_2.	Loss in fresh weight (per cent.).	Remarks.
2	42·8	18·7		(1) (2)
4	47·7	19·4		
6	45·8	20·0		(3)
		Interval of 19 days		
29	101	22·8	2	(4)
34	116	23·6		
41	171·7	24·0		
45	93·4	24·5		
48	95·3	25·0		
53	116·3	24·0		
58	124·4	24·6		
64	145·7	25·3		(5)
		Interval of 48 days		
121	210·0	27·0	6	
127	146·3	27·5		
133	141·8	28·0		(6)
138	129·1	29·3		
144	137·5	28·5	7	
		Interval of 1 day		
149	95·0	29·7		(7)
		Interval of 92 days		
241	140·0	27·9	12	(8)
		Interval of 2 days		
243	100·7	25·9		(9) (10)
		Interval of 78 days		
338	171·5	20·4	17·5	(11)
		Interval of 10 days		
356	192·0	21·9		
370	336·0	19·3		
377	168·0	20·1		(12)

(1) 24 hours allowed to reach temperature equilibrium in respiration chamber.
(2) Six apples in sample used, weighing 878 grams.
(3) All sound.
(4) A fresh sample of six apples used, weighing originally 898 grams.
(5) All sound.
(6) One apple commencing to rot and removed.
(7) All sound.
(8) Two more apples commencing to rot and removed after interval.
(9) At this point, the respiratory activity of a parallel series of five apples was 25·2.
(10) All sound.
(11) Two fresh apples used, weighing originally 278 grams.
(12) Both sound.

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The Influence of the Host Plant in Inducing Parasitism in Lucerne and Clover Nodules.

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[Plates 7–11.]

A.—*Introduction.*

The relationship of the nodule organism to its host plant has been much discussed, some authors regarding it as an instance of true symbiosis, while others regard the organism as a parasite to which the host plant offers a certain resistance. Failures to obtain inoculation of legumes with strains of nodule bacteria belonging to a different inoculation group may be regarded as examples of such resistance. Even where nodules are formed, the fixation of nitrogen and the benefit derived by the host plant varies according to the strain of the nodule organism concerned (Stevens (1) and Wright (2)). Some strains, while producing nodules, cause no increase in growth or nitrogen content in the host plant (3) and (4). It is uncertain whether the resistance of the host plant prevents the normal functioning of such strains, or whether they are actively parasitic on the nodule tissue.

Strains of the nodule organism thus differ in their relationship to the host plant. The behaviour of a single strain in the tissues may also be altered by the condition of the host plant. Thus, when *Vicia faba* is grown in a boron-deficient solution, the conducting tissue develops abnormally, so that the vascular supply to the nodules is either absent or incomplete. In such nodules the bacteria fix but little nitrogen and destroy the host cells in which they lie, although the same strain in healthy plants behaves normally and fixes appreciable amounts of nitrogen (Brenchley and Thornton (5)). It is thus possible experimentally to alter the relationship between the host plant and the bacteria, so that a strain of the latter which is normally beneficial to its host becomes actively parasitic. It was suggested that, in normal nodules, the bacteria derive their energy material from the carbohydrates conveyed to them along the vessels, but that in boron-deficient plants they are to a large extent deprived of their carbohydrates, owing to failure of the vascular supply, and derive energy by attacking the host protoplasm. If this hypothesis be correct, it

should be possible to induce the change from symbiosis to parasitism by cutting off the carbohydrate supply in other ways, for example by keeping the plants in darkness.

B.—Infection of Darkened Seedlings.

The following experiment was made to ascertain whether nodules could be formed on plants kept in the dark. Eighteen wide test-tubes were half filled with a mixture of equal parts of garden soil and sand and sterilised in the autoclave. Each was inoculated with a suspension of a 3-day old culture of the lucerne nodule organism. Twenty seeds of lucerne (*Medicago sativa*, L.) the coats of which had been sterilised by immersion in absolute alcohol followed by 0·2 per cent. $HgCl_2$, washed off with sterile water, were placed in each tube. Six tubes were kept in the light, six in darkness, and the remaining six in light until the seedlings showed the first true leaves open, after which they were kept dark. Germination took place in 7 days and, in the lighted seedlings, the first true leaves opened 5 days later. Three weeks after sowing 20 plants from each of duplicate tubes were examined. The development of nodules is shown in Table I.

Table I.—Effect of Light and Darkness on Nodule Formation.

	Nodules on 20 plants.			Mean length of nodules in mm.	Mean breadth of nodules in mm.
	Tube 1.	Tube 2.	Mean.		
Plant kept dark	3	4	3·5	0·25	0·25
Plants kept dark after true leaves opened	17	15	16	0·4	0·5
Plants kept in the light	42	51	46·5	1·0	0·75

Complete darkening of the plants almost entirely stopped the appearance of nodules. Darkening after the opening of the first true leaf greatly reduced nodule formation. Some of the nodules in this series probably appeared while the plants were still exposed to light, for although very few nodules develop before the true leaf opens, this rule is not absolute (Thornton (6)).

The following experiment was made in order to ascertain whether nodules continue to be formed after plants are placed in darkness. Sixteen wide test-tubes containing an agar medium, made up with plant nutrient solution.*

* The medium had the following composition :—K_2HPO_4, 0·5 grs. ; $MgSO_4 . 7H_2O$, 0·2 grs. ; NaCl, 0·1 gr. ; $Ca_3(PO_4)_2$, 2·0 grs. ; $FePO_4$, 1·0 grs. ; $FeCl_3$, 0·01 gr. ; agar, 10 grs. ; water, 1000 c.c.

were sterilised and each was sown with two lucerne seeds, the coats of which had been sterilised as described above. The seeds germinated in 3 days and the tubes were then inoculated with a 10-day old culture of the lucerne nodule organism. Two weeks later most plants had the second true leaf open and nodules had begun to form. 18, 19 and 28 days after germination, pairs of duplicate tubes were removed and placed in the dark. The number of nodules that appeared in these tubes before and after the tubes were darkened is shown in fig. 1. Darkening did not immediately stop nodule formation, one or

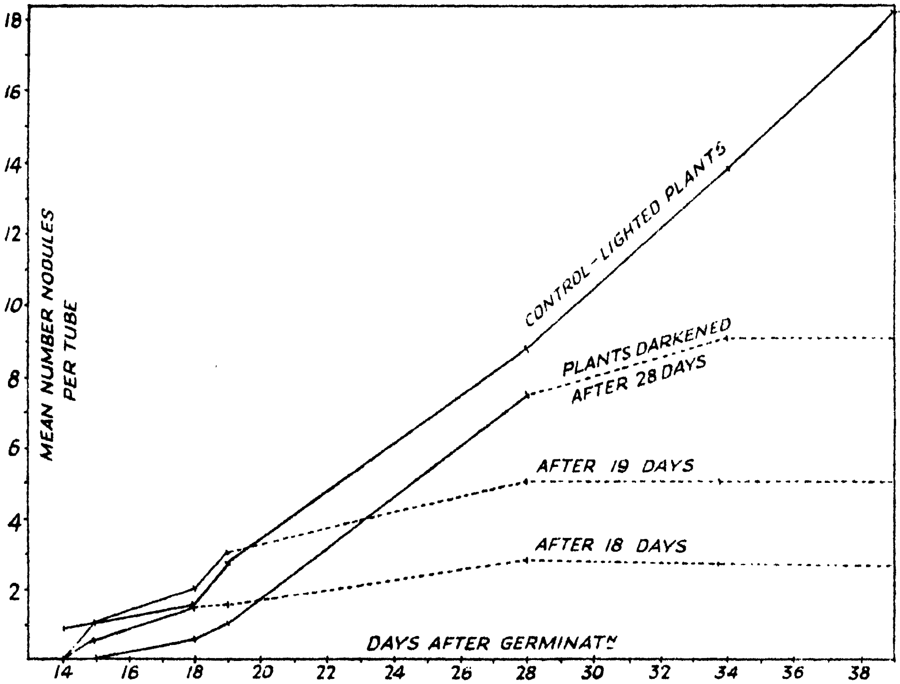

FIG. 1.—Appearance of Nodules on Seedlings grown in Light and in Darkness.

two nodules appearing after this was begun, but soon the process was completely stopped.

The arrest of nodule formation is presumably due to the absence of some factor which in lighted plants assists the development of nodules, since observations described below show that the protoplasm and cell walls of the host are not made more resistant to the nodule bacteria by darkening the plant. The plants apparently contained a small reserve of this factor at the time of darkening, no further nodules being formed when this was used up. The factor must be present in small quantities in the seed, since a few nodules

developed on plants grown entirely in the dark (Table I). When inoculated seedlings grown in the light are deprived of their cotyledons, the appearance of nodules is delayed until the true leaves are well developed (Thornton, 6). A substance essential to nodule formation is thus present in the cotyledons and is presumably also formed in the true leaves. It is perhaps the lack of this substance in darkened seedlings that stops the formation of nodules. The fact that cell division ceases in the roots of such seedlings suggests that the substance acts by furthering this process.

C.—*The Degeneration of Nodules on the Roots of Darkened Seedlings.*

The nodules on the roots of plants placed in the dark soon ceased to grow and remained small (Table I). The plants placed in darkness in the second experiment, bore some nodules which had appeared when the plants were lighted. These nodules, and others formed after the plants were darkened, were fixed in Bouin's fixative (7) and serial sections 5 μ thick were cut and stained with iron hæmatoxylin and orange G. These preparations were compared with others from a control series of nodules taken from plants kept all the time in the light.

The development of the nodule on normal lucerne seedlings grown in light is as follows. The bacteria infect a root hair, forming within it one or more "infection threads" containing the organisms in the short uniformly-staining rod stage. Infection threads penetrate cells as far as the inner layers of the cortex but do not penetrate the endodermis. Along the course of the threads the cells that have been penetrated and those adjacent to them become more densely protoplasmic, their nuclei increase greatly in size, and active cell division commences. The cell division thus begun produces a mass of meristematic tissue, characterised by protoplasmic cells with large nuclei (Plate 7, fig. 1). Through these the infection threads ramify.

Infected cells are sometimes seen in a state of division and in such cases the mechanism of the mitotic spindle does not appear to be damaged by the presence of the infection thread of bacterial zooglœa within the cell. The thread often grows towards and applies itself to the host cell nucleus, which, however, remains apparently undamaged by this.

When the nodule reaches a diameter of about 1 mm., the cells in the proximal region cease to divide (Plate 7, fig. 2). At the distal end, however, cell division continues, a meristem cap being thus formed, which causes the nodule to increase in length and to become roughly cylindrical. At about this time vascular strands grow out into the nodule cortex in the manner described in

detail in the case of *Vicia* by Brenchley and Thornton (5). Swelling of the infected cells and a rapid multiplication of the bacteria within them accompanies the growth of the vascular strands. Save in old nodule tissue the bacteria do not normally appear to injure the cells in which they lie, the nucleus of the host cell, and even the cytoplasmic strands running from it, being undamaged by them (Plate 8, fig. 1). Degeneration of the cell contents occurs only in old infected cells. The bacteria that lie in the cytoplasm show a progressive change in appearance, increasing in size and becoming banded (Plate 9, fig. 1). During the growth of the nodule the young cells in the inner layers of the meristem cap become infected by ramification of the infection threads, portions of which also persist in the proximal regions of the nodule and eventually play a part in its decay.

When lucerne seedlings are grown entirely in the dark such nodules as appear do not attain a diameter greater than 0·5 mm., and remain in the roughly spherical stage reached in normal nodules about the first or second day. Sections show that such nodules, when about 2 weeks old, consist of small cell tissue, the cells of which have ceased to divide (Plate 7, fig. 3). The cytoplasm has shrunk to a thin layer lining the cell walls and the nuclei are very small. These changes are not due to the action of the infecting bacteria, since they occur in other meristem tissue, such as young lateral roots. The infection threads are more numerous than in normal nodules. They show a greater tendency to swell out into zooglœal masses. They tend as usual to grow up to and apply themselves against the nucleus of the host cell; the latter however does not remain undamaged, but in many cells becomes surrounded by a mass of bacterial zooglœa and progressively degenerates, first losing its shape and structure and finally disappearing. This destruction of the nucleus is not seen in uninfected meristem tissue and is thus due to the presence of the bacteria, which have become actively parasitic (Plate 7, fig. 4).

On seedlings kept at first in light and then in the dark, nodules that had begun to develop in the light continued to increase in size for a few days and then stopped growing, cell division being arrested. In the younger portions of such nodules the infection threads tend to swell out into zooglœal masses and to attack the nuclei of the host cells, as was noted above. In the older swollen cell tissues the cytoplasm is destroyed, the nucleus shrivels up and vanishes, and the bacteria collect in dense masses in the cells. (Compare figs. 1 and 2, Plate 8.) The bacteria in such tissues change from swollen banded rods to much smaller rods and coccoid forms.

In nodules from darkened seedlings the bacteria do not remain confined to

the cells, but invade the intercellular spaces and the middle lamellæ of the cell walls. In young healthy nodules such invasion does not occur, the bacteria being entirely intracellular, except at the point where the infection threads cross the cell walls. In nodules from darkened seedlings, the points at which infection threads cross the cell wall become centres from which bacteria invade the intercellular spaces (Plate 8, fig. 4) and, by attacking the middle lamellæ of the walls (Plate 8, fig. 3), gradually cause the nodule tissue to disintegrate.

D.—*Decay of Old Nodules.*

The observation that in these abnormal nodules the bacteria tend to attack the middle lamella of the cell wall, has a special bearing upon the problem of the decay of old nodules on normal plants. In perennial legumes, such as lucerne, most of the nodules decay in the autumn, a fresh batch forming on young roots the following year. This decay takes place most rapidly in the interior of the nodule, so that decaying nodules are often hollow inside while the cortex is intact. Milovidov (8). in his observations on the histology of clover nodules, describes the older proximal region of the nodule as having the intercellular spaces invaded by coccoid rod forms, which eventually cause disintegration of the tissue. In lucerne, as in clover, coccoid rods invade the intercellular spaces and the middle lamellæ of the cell walls, in older parts of the nodules, towards the end of the season.

It was necessary to make certain that these bacteria were in fact the nodule organism, and were not contaminating organisms which had entered the nodule from outside. To decide this point, lucerne seedlings were grown in wide test-tubes containing the agar medium described above, which were sterilised in the autoclave and sown with seeds whose coats were also sterilised. When the seeds had germinated, 15 of the tubes were inoculated with a culture of the lucerne nodule organisms and 5 were left uninoculated, to serve as controls. These latter remained free from bacterial growth. The seeds were sown in April and, in the inoculated tubes, plentiful nodule development occurred. Towards the end of the summer the plants began to lose their leaves, and in September platings from nodules out of five of the tubes were made on lucerne-root extract agar and on nutrient agar. In no case did colonies other than those typical of the nodule organisms appear. A number of nodules from these same tubes were fixed in Bouin's fixative, paraffin sections were made and stained with iron hæmatoxylin and orange G.

In these old nodules the centre was usually hollow, the space being surrounded by collapsed and disintegrating tissue (Plate 9, fig. 4). The stages of this

disintegration could be followed by studying regions of different ages in the nodules. In the earliest stage, coccoid rods invade the intercellular spaces and the middle lamella of the cell wall. The invasion commences at the point where an infection thread crosses a cell wall. There are frequently pieces of infection thread persisting in the swollen cells containing bacteroids, and, in these threads, the bacteria remain in the form of coccoid rods. The bacteria that invade the middle lamella and the intercellular spaces multiply and remain in this form (Plate 9, fig. 2). The cell walls invaded by the bacteria swell out and eventually collapse (Plate 9, figs. 3 and 4).

Meanwhile progressive degeneration of the cell contents of the swollen cells and of their contained bacteroids takes place. The nuclei lose their shape and disintegrate and the cytoplasm shrinks to a small lump. The bacteria in the cytoplasm, which in younger tissue consist of banded rods (Plate 9, fig. 1) break up into darkly-staining granules (Plate 9, fig. 2) which eventually disappear or at any rate lose their staining properties.

In the final stages of decay, collapse and disintegration of the tissue results in the formation of a hollow space surrounded by fragments of cell walls and zooglœal masses of bacteria in the coccoid rod stage (Plate 9, fig. 4). When the nodule breaks up, it is presumably these bacteria which escape into the soil to infect fresh roots. Continuance of the race is therefore brought about by the descendants of the originally small numbers of bacteria which have persisted within the infection threads and which escape and multiply in the intercellular spaces. It is not brought about by the bacteria that have multiplied in the cytoplasm and carried out the nitrogen fixation, since these degenerate and apparently come to nothing. The life-cycle of the bacteria within the lucerne nodule may thus be represented diagrammatically as in fig. 2.

The process of decay was also followed in old clover nodules. For this purpose plants were grown aseptically as above described. Some tubes were kept uninoculated as controls, and these showed no signs of bacterial growth. Other tubes were inoculated with a pure culture of the clover nodule organism, and platings from the contents of these tubes, made at the conclusion of the experiment, showed only colonies typical of the nodule bacteria. Nodules were fixed and sections made and stained as described in the case of lucerne nodules.

In clover, as in lucerne, bacteria in the coccoid rod stage, derived from the old infection threads, invade the intercellular spaces and the middle lamellæ of the cell walls, from the points at which infection threads enter the cell

(Plate 10, fig. 3). Milovidov (8) states that cyst-like swellings, formed on the infection thread in young cells, act as reservoirs from which coccoid rods

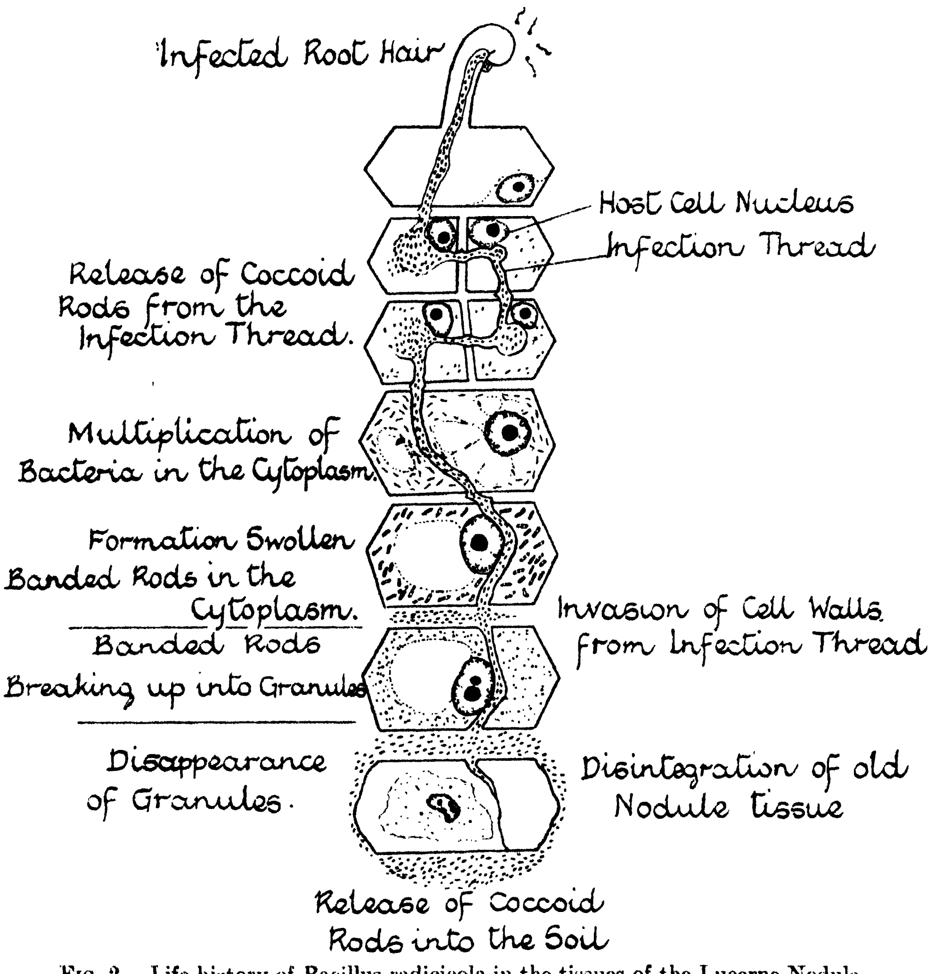

FIG. 2.—Life history of Bacillus radicicola in the tissues of the Lucerne Nodule.

invade the intercellular spaces in older tissue. The old infection threads themselves were centres from which the invasion took place in the material here studied. The infected cell walls swell up and the coccoid rods multiply therein (Plate 10, fig. 4). The bacteria in the cytoplasm, which become more swollen than in lucerne (Plate 10, fig. 1), break up into granules (Plate 10, fig. 2), which eventually lose their staining properties. The nuclei of the infected cells degenerate, losing their shape and structure and finally disappearing, and the cytoplasm shrinks to a lump in the centre of the cell.

E.—*Discussion.*

The behaviour of the nodule bacteria within the tissues is liable to be altered by the condition of the host plant and to change during the life of a single nodule. Three conditions are known under which a strain of nodule organism, known to exist normally within its host as a beneficial symbiont, will become actively parasitic and destroy the tissues of the same host. The first condition is induced by growing the plants in boron-deficient solution (Brenchley and Thornton (5)) so that the vascular supply to the nodule is absent or defective. The second condition is obtained by growing the host plant in the dark and thus preventing photosynthesis. In both conditions the normal carbohydrate supply to the nodule is cut off; in the first at the nodule itself and in the second in the leaves. It is natural to suppose that this shortage of carbohydrate is in both cases the cause of the similar change to parasitism, and that when no carbohydrate is brought to them, the bacteria obtain their energy by attacking the nucleus, cytoplasm, and cell walls of the host tissue.

The fact that the bacteria do not attack the host cells in normal young nodules is not, however, fully explained by this hypothesis. The host tissues provide a source of energy which they are able to utilise, since they do so when the carbohydrate supply is deficient. Why do not the bacteria in the healthy nodule increase up to the limit set by the total energy supply, consuming both the carbohydrates brought to them and also the host tissues? There is presumably some other factor which limits the bacterial population to a size which can obtain the needed energy from the carbohydrates normally supplied by the leaves. The air supply is a factor likely to limit the bacterial numbers in nodule tissue.

Observations showed that the functioning of nodules was closely dependent on good aeration. Seedlings were grown in wide test-tubes each containing a column of agar medium about 2½ inches deep. Under such conditions plants in some tubes develop nodules deeply imbedded in the agar, while in other tubes nodules either develop at the surface or else are given access in the air by shrinkage or cracking of the agar gel. Where the nodules are all deeply imbedded the plants remain weak, scarcely larger than uninoculated plants. Where the nodules develop with free access to the air, the plants grow much stronger (Plate 11, fig. 1). In the latter case there was appreciable nitrogen fixation, whereas the imbedded nodules produced no increase in nitrogen (Table II).

Table II.—Effect of Aeration of Nodules on Nitrogen Fixation in Lucerne Seedlings growing in Agar.

(Total nitrogen of seedlings plus agar medium.)

Tube number.	Total nitrogen.	Mean.
	mgs.	
Tubes with nodules exposed to air—		
1	4·22	3·53
2	4·13	
3	3·57	
4	3·12	
5	2·63	
Tubes with nodules imbedded in agar—		
1	2·06	1·88
2	2·00	
3	1·90	
4	1·55	
Uninoculated tube ...	2·26	2·26

In certain cases nodules, at first deeply imbedded, were subsequently exposed to the air by shrinkage of the agar; such nodules then began to benefit the plant, producing a sudden increase in growth and darkening of the leaves (Plate 11, fig. 2). Sections were made of nodules deeply imbedded which had not benefited the host plant, and of nodules growing at the surface whose host plants were growing strongly. In neither case were the bacteria in the young nodule tissue attacking the host-cell contents or invading the cell walls. Thus unhealthy and stunted growth of the plant did not in itself cause the bacteria to become parasitic. They did not attack the host tissue, because their numbers were limited by lack of air and the carbohydrates brought to them supplied sufficient energy material. In healthy functioning nodules, it is probably the air supply which still limits the bacterial population. But where the carbohydrate supply is cut down, energy material becomes the limiting factor, and the bacteria consequently consume all available material in the host tissues.

The third condition in which the bacteria become parasitic upon the host tissues is that brought about in old nodules. Here their behaviour bears obvious resemblances to the change to active parasitism induced by shortage of carbohydrate supply. In both cases they destroy nuclei of the host cells and invade the cell walls from the infection threads. Here also the change in relationship between the bacteria and the host tissues is perhaps caused by a failure of the carbohydrate supply within the host cells, due either to lessened

photosynthesis as the season advances or to some failure in the translocation of carbohydrates into the nodule.

Summary.

1. When inoculated lucerne seedlings are placed in the dark, the formation of fresh nodules is soon stopped and nodules already formed soon cease to grow in size. This is associated with, and probably due to, the cessation of cell division throughout the root.

2. In such nodules, the bacteria become parasitic upon the host tissues, destroying the cytoplasm and nuclei of the infected cells, and invading the cell walls and intercellular spaces.

3. In old nodules on lucerne and clover plants growing in the light, the bacteria behave similarly. The swollen forms in the cytoplasm, after the destruction of the host nucleus, break up into granules which eventually disappear, while coccoid rod forms from the old infection threads invade the cell wall and intercellular spaces, causing the nodule tissue to disintegrate.

4. It is suggested that in both these cases the change in behaviour of the bacteria is due to lack of carbohydrate, and that, where this is limited, the bacteria derive their energy from the host tissue.

5. This hypothesis implies that, in normal nodule tissue, some other factor, perhaps air supply, limits the bacterial population and thus prevents them from attacking the host tissues.

6. Observations on lucerne seedlings growing in agar show that where the air supply is inadequate, the nodules do not function normally, although these conditions do not cause the bacteria to injure the nodule tissue, the carbohydrate supply not being the factor limiting their growth.

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DESCRIPTION OF PLATES.

PLATE 7.

FIG. 1.—Young lucerne nodule on normal seedling grown in the light, showing protoplasmic cells and large nuclei. Dividing cell (*m*), stele (*stl*), nodule cortex (*ct*). (× 330.)

FIG. 2.—Slightly older lucerne nodule on normal seedling, showing differentiation of tissues into meristem cap (*ms*), swollen cell region (*sw*), and nodule cortex (*ct*). (× 200.)

FIG. 3.—Nodule from lucerne seedling grown in the dark, showing disappearance of cytoplasm, shrinkage of nuclei and great increase in quantity of infection threads (*st*). (× 330.)

FIG. 4.—Part of the nodule shown in fig. 3 showing nuclei (*d*), being destroyed by bacterial zooglœa (*z*). Uninjured nuclei (*n*). (× 1000.)

PLATE 8.

FIG. 1.—Swollen infected cells in a normal nodule from a lucerne seedling grown in light, showing large nuclei (*n*), and cytoplasmic strands (*cs*), uninjured although the cytoplasm is filled with bacteria. (× 1000.)

FIG. 2.—Swollen infected cells in a nodule from a lucerne seedling placed in the dark, showing destruction of the cytoplasm, and the shrunken and distorted nucleus (*n*). Masses of bacteria (*bac*), infection thread (*st*). (× 1000.)

FIG. 3.—Nodule tissue from lucerne seedling grown in the dark, showing invasion of the cell walls by the bacteria. (× 1000).

FIG. 4.—Cells from the same nodule as fig. 3 showing infection of the cell wall (*cw*), from an infection thread (*st*). (× 1000.)

PLATE 9.

FIG. 1.—Young swollen cell tissue from a nodule on lucerne grown in the light, showing bacteria in the banded rod stage. (× 1000.)

FIG. 2.—Swollen cell tissue from an old lucerne nodule (plant grown in light), showing that the bacteria in the cytoplasm have broken up into granules (*gn*), which gradually disappear (*gns*), while the cell walls have become infected with coccoid rods (*cw*). (× 1000.)

FIG. 3.—Tissue from a similar old lucerne nodule showing swelling of the infected cell walls (*cws*), and remains of cytoplasm (*gns*). (× 1000.)

FIG. 4.—Tissue from the centre of the same nodule showing collapse of weakened cell walls (*cws*). Hollow space in the centre of the nodule (*sp*), bacterial zooglœa (*bac*). (× 1000.)

PLATE 10.

FIG. 1.—Young swollen cell tissue from clover nodule, showing "bacteroids" (*bac*). Host cell nuclei (*n*). (× 1000.)

FIG. 2.—Swollen cell from old clover nodule showing bacteria breaking up into granules. (× 1000.)

FIG. 3.—Swollen cell from old clover nodule showing bacteria from infection thread (*st*), invading the cell wall (*cw*). Remains of host cell nucleus (*n*). (× 1000.)

FIG. 4.—Tissue from old clover nodule showing invasion of cell walls. (× 1000.)

PLATE 11.

FIG. 1.—Lucerne seedlings grown in agar. (*a*) Uninoculated controls, (*b*) Inoculated plants whose nodules are imbedded in agar, (*c*) Inoculated plants some of whose nodules are exposed to the air by shrinkage of the agar.

FIG. 2.—Lucerne seedlings grown in agar. (*a*) Uninoculated controls, (*b*) Inoculated plants whose nodules were for some time imbedded but have since become exposed to air, (*c*) Inoculated plants some of whose nodules have been exposed to air from an early stage.

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The Formation of Lactic Acid in Desiccated Amphibian Muscles.

By E. C. SMITH and T. MORAN.

(Communicated by Sir William Hardy, F.R.S.—Received December 18, 1929.)

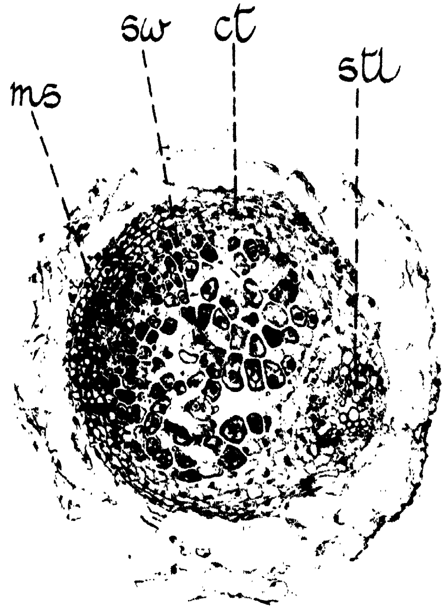
The present paper is the third of a series dealing with the effects of freezing and drying on living amphibian muscle (1, 2). In this paper it is shown that simple drying of muscles causes the production of lactic acid. in amount identical with that produced by the corresponding degree of freezing (2). This finding confirms the view expressed in the earlier communications that the effects of freezing and drying are identical, the determining factor in each case being the removal from the muscle of a certain amount of water.

Experimental Procedure.

To ensure even drying throughout, it was necessary to use a thin muscle of regular geometrical form. The sartorius muscle of the frog was therefore chosen. The frogs were precooled for some hours at zero. The muscles were then carefully removed at their insertions, wiped on filter paper, and each individual muscle suspended by a silk thread of known weight (see later) in a stoppered bottle, over 0·72 per cent. sodium chloride solution. After standing at 0° C. for 24 hours, to allow of the removal of the lactic acid formed during the manipulation, the muscles were weighed, and rapidly dried by being suspended in bottles over solid calcium chloride. It has already been shown that this method of drying produces a uniform state throughout the muscle (1, p. 187). They were removed at intervals for weighing.

When the required amount of drying had been attained the muscles were transferred to bottles, in which they were suspended over that solution of

FIG. 1.



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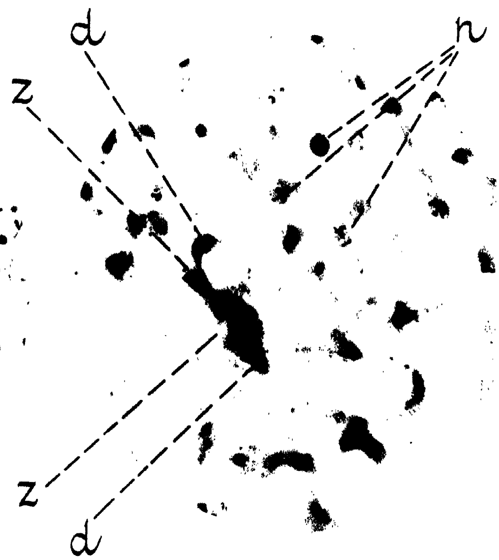

FIG. 4.

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Fig. 1.

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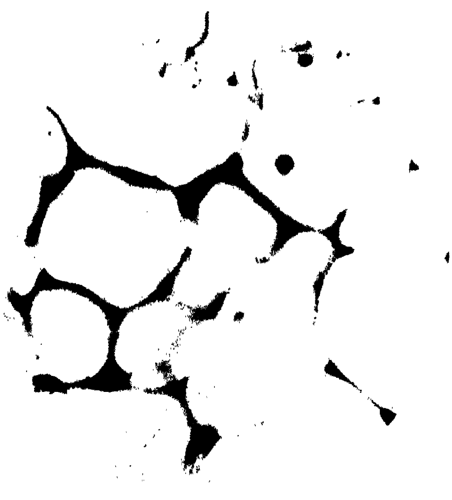

Fig. 3.

Fig. 4.

Fig. 1.

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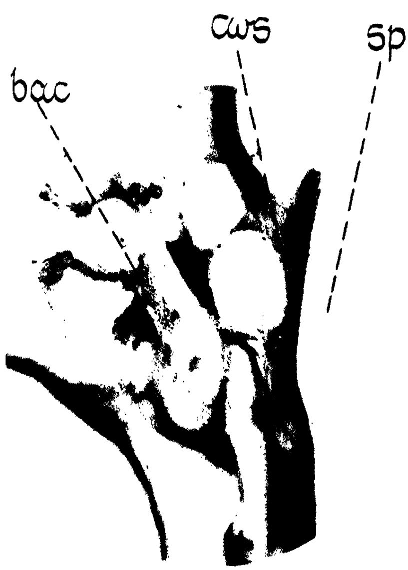

Fig. 4.

Fig. 1.

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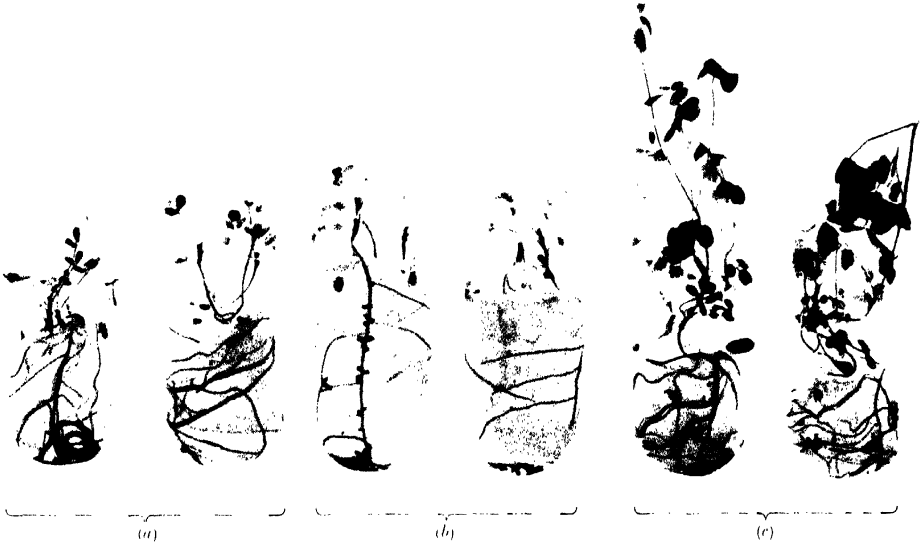

Fig. 1.

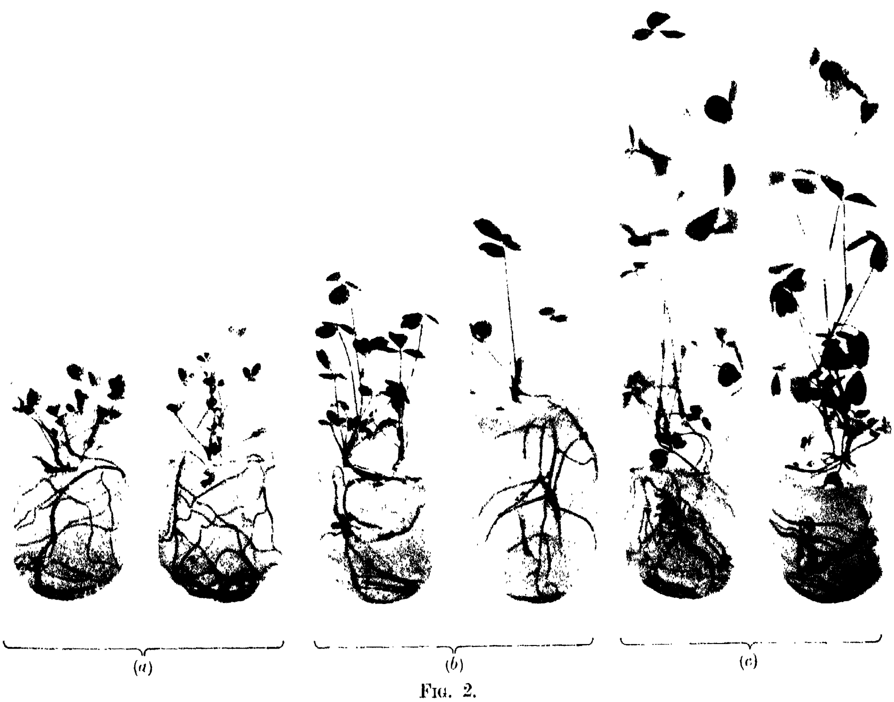

Fig. 2.

calcium chloride whose vapour pressure would maintain the weight of the muscles constant. With experience it was possible to get the required degree of desiccation with only two weighings, and to adjust the concentration of the solution of calcium chloride practically at once.

For each determination of lactic acid six muscles were used. Only those experiments in which the mean weight of the six muscles varied less than 2 per cent. in 48 hours were recorded. The temperature of storage was in all experiments 0° C., and the time of storage 48 hours from the commencement of drying.

In calculating the degree of drying of the tissue it was necessary to take into account the drying of the silk threads used for suspension. In the process of dissection of the muscle, these threads were unavoidably moistened to a slight extent with lymph. The mean loss in weight on drying of a number of threads similarly contaminated was determined, and the necessary correction applied in calculating the drying of the muscles.

The batches of six dried muscles, at the end of the storage period, were ground up in a small quantity of ice-cold trichloracetic acid, and the lactic acid determined in the protein-free filtrate by the Clausen method, with the precautions already described (2).

In fig. 1 are plotted the concentrations of lactic acid, expressed in grams per cent. of the fresh weight of the muscle, reached at the end of 48 hours' storage

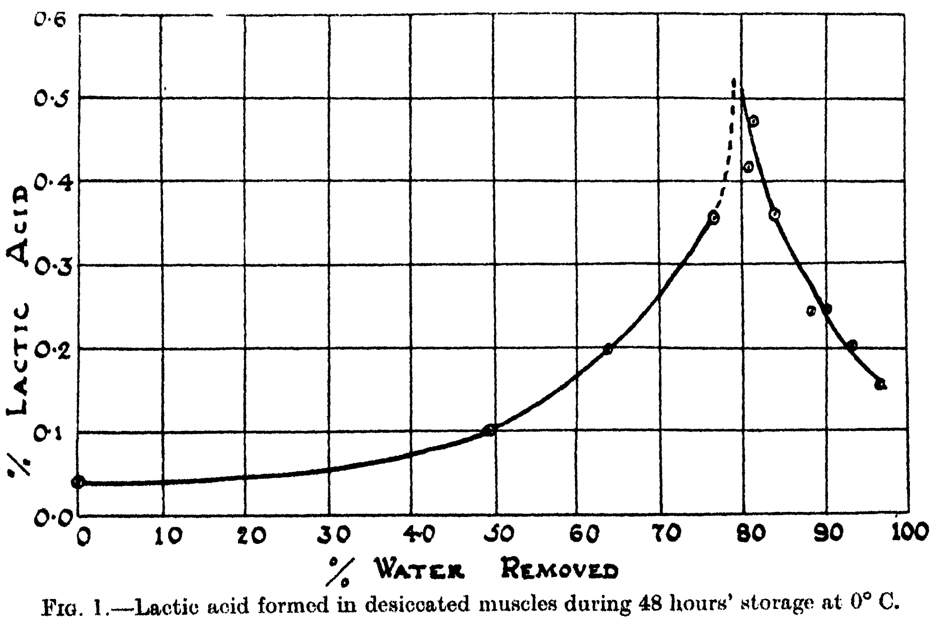

Fig. 1.—Lactic acid formed in desiccated muscles during 48 hours' storage at 0° C.

at 0. The drying of the tissue is expressed as the amount of "available" water removed from the tissue. For the present purpose, "available" water is taken to be the amount of water removed from the muscle by drying *in vacuo* to constant weight over phosphorus pentoxide. The value (77 per cent.) represents the mean available water content of 12 sartorius muscles.

Quantitatively, the form of the curve in fig. 1 resembles very closely the curve obtained in the freezing experiments on the gastrocnemius muscle, when the removal of water by freezing is assumed to be the same as that of a solution of salt isotonic with muscle (*cf.* Smith (2), fig. 2). There are, however, slight differences in scale and in the position of the curves. As these differences were considered to be due, in part at least, to differences in the behaviour of gastrocnemius and sartorius muscles, the freezing experiments were repeated, using sartorius muscles in place of the gastrocnemius. A parallel series of drying experiments was carried out at the same time, and in order that the values obtained in the respective series should be exactly comparable, the muscles of the one side were used for the freezing experiments and the corresponding muscles of the other side for the drying experiments. The storage conditions were so chosen that the water removed as ice from the frozen muscles was as nearly as possible equal to that removed from the dried muscles.

The Temperature Coefficient of Lactic Acid Production.

To compare the lactic acid present in dried muscles stored at 0° C. with frozen muscles stored at a lower temperature, it was necessary to know the effect of temperature within the narrow range involved upon the rate of accumulation of the acid. The limits of the range were 0° C. and the freezing point of the most desiccated muscles, which was − 5·5° C.

It was not thought necessary to explore the whole of so narrow a range, indeed, it was not possible, because the choice of a lower limit of temperature for these determinations was restricted by the necessity for having a measurable yield of lactic acid. The temperature which gave the largest range compatible with a reasonable yield was − 3·5° C., which is the freezing point of muscles which have lost 85 per cent. of their water.

In none of the four experiments carried out was there a difference of more than 3 per cent. between the lactic acid concentrations of muscles stored at the higher and lower temperatures respectively. This difference is well within the experimental error of determination of lactic acid. In each experiment the sartorius muscles of six frogs were used, dried to 80–90 per cent. water removal, those of the one side being stored at 0° C., the corresponding muscles

of the other side at $-3 \cdot 2°$ C. The results are tabulated below. The lactic acid concentrations are expressed as grams per cent. of the fresh weight of the muscle.

Table I.—Lactic Acid Concentrations of Dried Muscles.

	Temperature of storage.		Water removal percentage.
	0° C.	−3·2° C.	
1	0·297	0·290	90
2	0·231	0·237	90
3	0·231	0·231	90
4	0·383	0·370	80
Mean	0·286	0·282	

The muscles at 80 per cent. loss of water were overcooled by about $-0 \cdot 5°$ C. The mean production of lactic acid in muscles dried at 0° C. to 80–90 per cent. and stored at $-3 \cdot 2°$ is identical, within the limits of accuracy of measurement, with that in muscles similarly dried but stored at 0° C. The temperature coefficient of lactic acid formation over this narrow range of temperature may, therefore, be taken as unity, and it is not necessary to take into consideration the difference between the temperature of storage of the dried and frozen muscles in the following experiments.

Each of the figures in Table II represent the mean lactic acid concentration of the sartorius muscles of 12 frogs. In actual fact the figures for the dried muscles fall quite accurately on the curve in fig. 1, and are therefore not separately plotted. The values for the production in the frozen state are plotted in the lowest curve in fig. 2.

Table II.—Correlation of the Effects of Drying and Freezing.

	Temperature of freezing.	Lactic acid per cent.	Per cent. removal of water.	Lactic acid per cent.
5	−1·4	0·173	64·0	0·197
6	−2·0	0·412	83·3	0·526
7	2·1	0·363	76·5	0·354
8	−3·2	0·438	80·0	0·414
9	−5·3	0·238	93·0	0·203

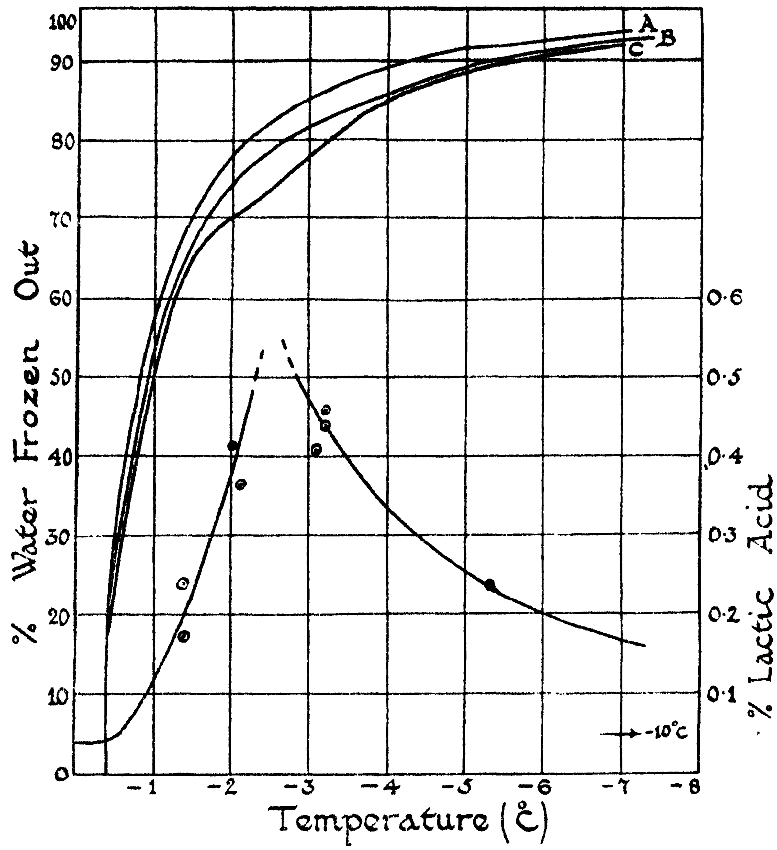

FIG. 2.—Formation of lactic acid in frozen amphibian muscle and relation between freezing and drying.

These values enable one to calculate the percentage of water removed from a muscle at any temperature between the freezing point and — 6° C., if it be assumed that the rate of production of lactic acid is a pure function of the degree of desiccation.

For example, the lactic acid produced at —4° C. is 0·33 per cent., and in the curve in fig. 1 this corresponds to a removal of 86 per cent. of water. By such comparisons made between —0·42 and —6° C. the curve B in fig. 2 was constructed. It gives the percentage removal of water as ice in terms of temperature.

Previous estimates of the amount of water removed at any given freezing temperature have been made by assuming that the aqueous phase of muscle behaves in the same way as the solution of sodium chloride with which it is isotonic, at zero and in the resting condition. The behaviour of such a solution

(0·72 per cent.) is represented by the curve A in fig. 2. There is a distinct divergence between the two curves A and B. In the construction of curve A, however, no account has been taken of the effect on the osmotic pressure of the muscle of the lactic acid produced during the process of removal of water. If the lactic acid so formed is assumed to be present in a completely dissociated condition, a third curve (C, fig. 2) can be constructed for temperatures between —0·42 and —8° C. which represents the water removed from a salt solution isotonic with muscle, but containing, in addition, the equivalent of the lactic acid present after 48 hours' storage at temperatures in that range. The curve B is intermediate between A and C.

In all probability, the lactic acid formed is in part undissociated, or, in combining with base releases incompletely dissociated acids, so that the full effect of the increase in concentration of lactic acid is not exerted on the osmotic pressure. In this case a curve of the form of curve B, intermediate between A and C, is to be expected. The fact that such a curve has been found experimentally would appear to justify the assumption made in constructing the curve, namely, that the formation of lactic acid in frozen muscle is due to the removal of water, and to this factor alone.

Discussion.

There appears to be a sharply defined temperature, slightly below —2° C., at which an irreversible change, involving the death of the tissue, takes place. The change must be so rapid as to be almost instantaneous, for on plunging a muscle into liquid air, there is no recovery on thawing, however rapidly the cycle of operations may be carried out. At freezing temperatures above —2° C. death eventually occurs, the length of time required being longer the less severe the freezing, so that a time factor is involved in this region (1, p. 182). Interest mainly centres, however, in the event which takes place instantaneously at a temperature between —2·0 and —2·5° C. It coincides with that degree of desiccation which produces the maximum rate of accumulation of lactic acid.

The data may be summarised as follows :—

	From freezing data.	From drying data.
(1) Point of instantaneous death	**Slightly below —2° C. (corresponding to a water removal of 75+ per cent.)**	At 78 per cent. water removal.
(2) Point of maximum rate of lactic acid formation	Between —2·2 and —2·5° C. **(corresponding to a removal of 79 per cent.)**	At 79 per cent. water removal.

There can now be no doubt that the excess production of lactic acid is due to desiccation, whether by drying or by freezing. The question therefore remains, how does desiccation act?

It is necessary to consider the two limbs of the curve in fig. 1 separately. Loss of water at first may be supposed merely to alter the rate of working of the machine, for on thawing it is found to be undamaged (1, p. 178; 2, p. 203). The descending limb suggests either that the mechanism of production is being destroyed, or that production is being delayed by increasing viscosity or by the removal from the working system of a necessary constituent.

There is no evidence to support the first possibility, for, on thawing, lactic acid is produced at a rapid rate. The effect of increased viscosity will certainly be to delay production, but not to the extent indicated by the abrupt change in the direction of the curve which occurs at 80 per cent. removal of water.

Consideration of the third possibility led us to examine the available data in search of a constituent of the plasma which might have a eutectic point in this critical region. It was found that the following salts were reputed to have eutectic points in this neighbourhood:—

Na_2HPO_4	$-0 \cdot 45°$ C.	(Landolt Börnstein).
$NaHCO_3$	$-2 \cdot 6°$ C.	(Landolt Börnstein).
K_2HPO_4	$-2 \cdot 0°$ C.	(Stiles (3)).

In the presence of salts of lower eutectic point these temperatures, referring to the single salts in pure solution, must necessarily be modified. The salt Na_2HPO_4 will illustrate the considerations that arise.

The Possibility of Precipitation of Na_2HPO_4 *in Frozen Muscle.*

Sodium phosphate in aqueous solution has its eutectic point at $-0 \cdot 45°$ C. (Landolt-Börnstein). In common solution with other salts the temperature at which this salt will be thrown out of solution is dependent on:—

(1) The concentration of sodium phosphate in the unfrozen solution.

(2) The concentration of other crystalloids of lower eutectic point.

(3) The effect on the solubility of sodium phosphate of the other bodies present.

Ignoring for the moment the third factor, the known relationships in muscle will enable a provisional temperature of precipitation to be decided upon.

According to Katz (4), the sodium content of frog's skeletal muscle is 0·0552 gms. per cent. (At a pH of 7·1 the sodium is probably completely

ionised, hence $[Na^{\cdot}]$ may be taken as 0·0552 gms. per cent.) The soluble phosphorus is 0·1523 per cent. (Katz), which would correspond to a concentration of HPO_4'' of 0·47 per cent. Only a small fraction of this can be present as the free ion. Eggleton (5) gives 0·020 per cent. P (0·062 per cent. HPO_4'') as the concentration of inorganic phosphate in resting frog's muscle. At *p*H 7·1 the ratio of H_2PO_4' to HPO_4'' in phosphate buffer mixture is 1 : 1·8 (6), hence if the whole of the inorganic phosphate were ionised this would amount to 0·04 per cent. of HPO_4'' or an effective concentration of 0·06 per cent. Na_2HPO_4.

The removal of water from muscle on freezing results in a gradual concentration of the salt solution. At any temperature the actual concentration can be calculated. For the above solution of Na_2HPO_4 the rate of concentration with temperature is plotted in curve A, fig. 3.

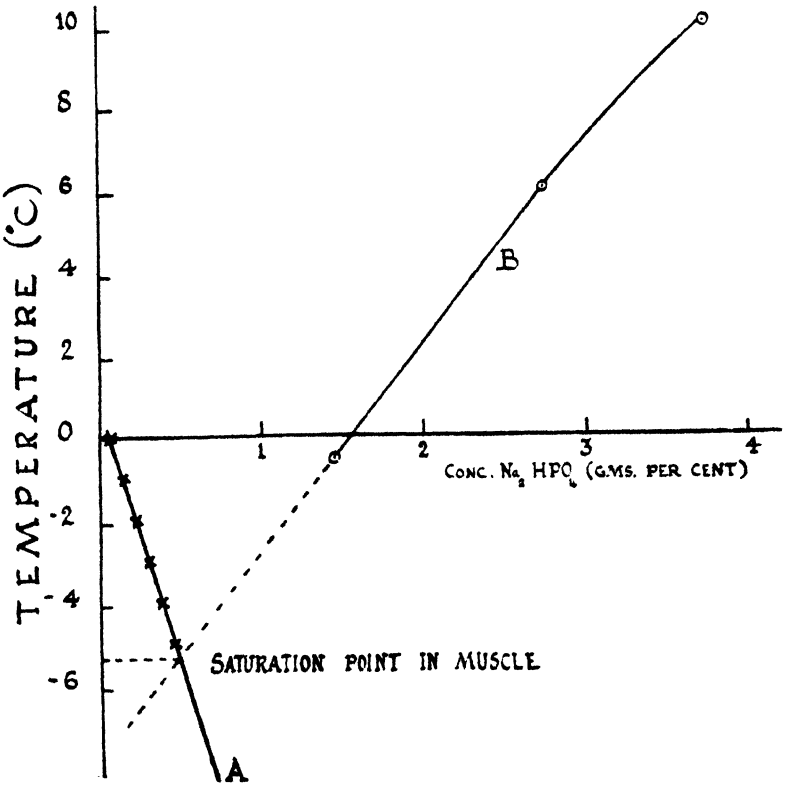

FIG. 3.—Concentration of Na_2HPO_4 in liquid phase of frozen amphibian muscle.

Plotting, in curve B, the decline of solubility of Na_2HPO_4 with temperature (and assuming that the curve may be extrapolated below the eutectic point,

as in the dotted portion of the curve) it is seen that the muscle plasma would not become saturated with sodium phosphate until a temperature of approximately —5° C. is reached. The eutectic point (—0·45) in pure solution is, therefore, no immediate guide to the behaviour of the salt in a complex mixture.

Taking into consideration now the third point—the presence of these salts might so affect the solubility curve B that saturation would occur at a higher temperature than —5°. It has been found, however, that a salt composed of unlike ions (*e.g.*, KCl) has the effect of increasing the solubility rather than diminishing it. Thus at 10° C. the solubility in 25 per cent. KCl solution was found to be 5·2 per cent., as against 3·75 per cent. in pure water, and at —2·6° C., 4·5 per cent., as against the extrapolated value of 1·6 per cent., so that it is to be expected that the saturation point is not reached in muscle until a considerably lower temperature than — 5°. Although these data are somewhat circumstantial, it would appear that no separation of sodium phosphate can occur in the critical region.

The case for sodium bicarbonate is of the same nature. The eutectic point in muscle plasma could not possibly be reached above —15° C. With regard to K_2HPO_4, the statement that the eutectic point is —2° C. could not be confirmed. It is certainly below —13° C. but is not well defined, probably owing to the separation of a more basic salt and the retention in solution of a more acid salt (*cf.* Mellor (7)).

The possibility cannot be excluded that a separation of calcium or magnesium salts takes place as the concentration of the plasma is increased. The state of these salts in physiological fluids is, at present, too obscure to admit of discussion. Until more is known of their behaviour one must be content with the purely negative conclusion that the peak in the production curve and the death of the muscle cannot be correlated with any known change in the salt constituents of the plasma.

Summary.

1. Drying and freezing are identical in their effects on the formation of lactic acid in amphibian muscle.

2. The peak in the production curve which occurs at —2·2° to — 2·5° C. or at 79 per cent. water removal, is apparently coincident with the point at which instantaneous death occurs in the muscle.

These two events cannot be associated with a sudden change in the concentration of any constituent of the plasma of which the behaviour in physiological solution is known.

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The "Nucleal Reaction" in Apanteles sp., *with Special Reference to the Secondary Nuclei and the Germ-Cell Determinant of the Egg.*

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[Plate 12.]

Introduction.

The question of the origin, nature and significance of the secondary nuclei and the germ-cell determinants, especially in the unfertilised eggs of the Hymenoptera, has been a subject of diverse opinion amongst the various observers. It is a curious fact that the secondary nuclei have not been found, except in Hemiptera, Lepidoptera, Coleoptera, Hymenoptera and Diptera, where they have been recorded by workers such as Blochmann (1), Stuhlmann (21), Korschelt (12), Paulcke (19), Marshall (18), Loyez (14), Hegner (8-11), Buchner (2) and Gatenby (6).

Some workers (Blochmann, Stuhlmann) maintain that they arise by a process of budding from the germinal vesicle, some (Loyez, Hegner, Buchner and Gatenby) that they are formed from extrusions of chromatin from the nucleus, and some (Korschelt) again that they are wandering granules from the nuclei of the surrounding follicular cells. In Apanteles, which was worked upon by both Hegner and Gatenby, the secondary nuclei are found disposed around

the periphery of the egg. According to the latter author. who worked with the most up-to-date cytological technique, some of these nuclei simulated the appearance of the head-nucleus in every respect, except the size. In Myrmicina (Buchner) their disposition around the head-nucleus and their staining behaviour lead one to believe that they are emissions of chromatin from the nucleus.

In Apanteles no such relationship has been found with the head-nucleus, and the question naturally arises whether they are emissions from the germinal vesicle at all. However a few broad facts concerning the so-called secondary nuclei in the oogenesis of many insects will be mentioned :—

1. Their origin is uncertain.
2. They may be disposed in various ways—either scattered in the oocyte, or round its periphery or round its germinal vesicle.
3. They have the power of growth.
4. They stain exactly like chromatin with basic stains.
5. They have not been observed to divide mitotically.
   (Amitotic division and budding of the secondary nuclei have, of course, been observed by Buchner in the Ichneumon Rhyssa.)
6. In every case, they disappear before the maturation period.

The origin of germ-cell determinants is alike unknown. They have been variously derived by different investigators—some from the nucleus consisting of nucleolar or chromatinic material, some from the cytoplasm arising as a product of differentiation, some from the nurse-cells (its cytoplasm or the nucleus). some from bodies of a mitochondrial nature, and some from the secondary nuclei themselves.

For a complete account of this structure the reader is referred to the works of Hegner (8–11) and Gatenby (6). It may be sufficient here to state the conclusions arrived at by Gatenby regarding this structure in Apanteles :

1. It consists of a basophil albuminous proteid which originates as a dense area at the posterior pole of the oocyte.
2. It contains no glycogen or fat.
3. It has no relationship whatever with the other two elements of the cytoplasm, viz., the mitochondria and the Golgi bodies.
4. It has no connection with the secondary nuclei, or the yolk-spheres, or the germinal vesicle.

It is not the object of this paper to verify the results of the various workers.

but with Feulgen's (5) test for chromatin at our disposal. I believe it is possible to disprove the claims of those workers who hold that these basophilic structures are chromatinic in nature.

This work was undertaken at the suggestion of Prof. Gatenby, to whom my thanks are due.

Summary of Feulgen's Reaction for Test of Chromatin.

Biochemists maintain that the chromatin of the nucleus, whether animal or vegetable, consists mainly of a salt of nucleic acid with a protein base. Hydrolysis of this nucleic acid shows that it is again composed of simpler forms, such as phosphoric acid, purine and pyrimidine bases, and carbohydrate. The difference between the various nucleic acids lies in the number of carbon atoms constituting this carbohydrate molecule. Thus in some it is a five-atom group (pentose-containing nucleic acid), in others it is a six-atom group (hexose-containing nucleic acid).

The number of carbon atoms constituting the carbohydrate molecule is important, for it is on this fact that the nucleal reaction of Feulgen depends. The thymonucleic acid, which is supposed to be present in all cells whether animal or vegetable (except the yeast cells) belongs to the hexose-containing nucleic acid type. Often this thymonucleic acid may exist in combination with a pentose-containing nucleic acid in the tissue cells.

By a very careful hydrolysis of the thymonucleic acid, Feulgen has been able to obtain an acid devoid of the purine bases, but containing the phosphoric acid and the other parts of the molecule. The effect of such a hydrolysis is, according to Feulgen, supposed to set free the aldehydic groups or radicles (formerly combined with the purine bases) which react with fuchsin-sulphurous acid, forming a dye, exactly as an aldehyde behaves towards the same reagent. In other words, the well-known Schiff's test for aldehydes here finds application for the demonstration of the chromatin of the nucleus, which is dyed characteristically with fuchsin-sulphurous acid after mild acid hydrolysis. Hydrolysis of the pentose-containing nucleic acid, on the other hand, does not expose any such aldehydic radicle, and is therefore unable to give the Schiff's reaction. Thus a nucleus with a pentose-containing nucleic acid will fail to give the Feulgen's reaction.

A few essential facts of this important reaction will be detailed here. For a fuller account and the preparation of the various reagents, the reader is referred to the original article of Feulgen (5), Ludford (17), Cowdry (3) and 'Micro-

tomist's Vade-Mecum' may also be consulted. Broadly, the whole reaction may be divided into a series of stages :—

(*a*) Proper fixation in a fixative not containing any aldehyde (*e.g.*, formol) or an oxidising agent (*e.g.*, chromic acid).
(*b*) Washing-out the plasmal (an aldehyde), derived as a result of $HgCl_2$ or any acid fixation, from the tissue by means of alcohol.
(*c*) Hydrolysing the thymonucleic acid contained in the tissue by means of normal HCl at 60° C.
(*d*) Staining in fuchsin-sulphurous acid (for method of preparation see either Feulgen or Ludford).
(*e*) Washing-out the excess of fuchsin-sulphurous acid in several changes of SO_2-water.
(*f*) Washing in distilled water, clearing and mounting.

The various pitfalls in performing this reaction should be guarded against; these may arise from the following sources :—

1. Retention of plasmal in the cell.
2. Introduction of the salts of weak acids (*e.g.*, sodium acetate) or ions (*e.g.*, Ba) that will precipitate SO_3-ions, into the fuchsin-sulphurous acid.
3. Using fixatives containing an aldehyde or an oxidising agent.
4. Failure to remove the excess of fuchsin-sulphurous acid by means of SO_2-water (for some preparations, especially vegetable, often obstinately retain the reagent).
5. Failure to prepare the fuchsin-sulphurous acid containing the right excess of SO_2 and HCl in order to maintain the sensitivity of the reagent.
6. Performing the reaction in a room smelling of formalin.
7. Passing the slides through alcohol containing traces of formaldehyde.

Personal Investigations.

Fig. 1 is the earliest stage of a growing oocyte of Apanteles, depicted in Plate 12. The secondary nuclei are visible as small structures, each with a granule in the centre. This granule does not take up the purple coloration when the sections are subjected to Feulgen's method of treatment for the demonstration of chromatin; but light green, used as counterstain, stains it fairly deeply. The nucleus at this stage shows a few granules of chromatin coloured purple. In fig. 2, a fully grown oocyte, the secondary nuclei are still seen, stained green; but the germinal vesicle, which is at the end of the prophase of hetero-

typic division, does not show any sign of chromatinic granules characteristically stained.

In the next figure, the nucleus shows a few granules stained green, with light green ; in other respects it resembles the oocyte in fig. 2. In fig. 4, a stage slightly later than fig. 2, the secondary nuclei are very rare and the nucleus is without any recognisable chromatin elements. Fig. 5 is a stage in the process of maturation where the chromosomes are seen coloured purple.

In none of these stages can any trace of the germ-cell determinants be found. It seems that they suddenly appear, after the maturation division is completed, as a differentiation product of cytoplasm, or its elements, other than the mitochondria and the Golgi bodies. In materials fixed in Petrunkewitsch and stained in iron-hæmatoxylin, they are either structures of homogeneous composition or structures with a colourless matrix, studded over with deeply-stained granules. In eggs which are about to be laid (represented in fig. 6) they often differentiate into two zones—an outer composed of more colourable substance, and an inner almost colourless. Feulgen's test for chromatin failed to stain them even slightly, and in sections, not stained with light green, they were hardly recognisable from the surrounding cytoplasm ; but with the use of the counterstain, and with the knowledge of their position, they could be identified as very lightly-stained bodies at the posterior region of the egg.

At first it seemed probable that perhaps the germ-cell determinants lost some of their basophilic constituents during the process of acid hydrolysis, thus making them scarcely distinguishable from the surrounding protoplasm. With a view to investigating this, some sections were hydrolysed in the acid, but instead of staining them with fuchsin-sulphurous acid, they were treated with iron-hæmatoxylin. Sections thus stained showed the germ-cell determinants just as well as unhydrolysed sections.

The chromatin in the female pronucleus of the fully-matured eggs always stained deeply with the nucleal reaction ; so also the chromatin in the nuclei of the nurse-cells. In fig. 5, the chromatin granules in the nuclei of the follicular cells were very finely divided, but even then they retained their individual identity. No evidence was obtained of chromatinic emissions from the follicular cells into the oocytes or the mature eggs.

Discussion.

Since the discovery by Feulgen that chromatin (containing a nucleic acid of the type of thymonucleic acid) can be stained specifically by means of fuchsin-

sulphurous acid preceded by a mild acid hydrolysis, workers have found the method very convenient for the demonstration of the amount and disposition of chromatin present in a particular cell. Added to this, this method has been very useful in distinguishing certain basophilic structures of true chromatinic origin from other basophilic granules found in the cytoplasm of the cell. In the gametogenesis of many animals similar structures have been found in the cytoplasm, but their nature and origin have remained a subject of dispute amongst the various workers. In cases where an origin has been traced to the nucleus (not to the Golgi or the mitochondrial elements) observers have maintained that they arise either from the chromatin of the nucleus or from the nucleolus. Though the former presumption has since been discarded, on account of its being at variance with the chromosome theory, the latter has found acceptance among certain modern cytologists, of whom Ludford and Gatenby may be mentioned.

Even though little work has been directed to the study of gametogenesis by the application of this nucleal reaction, it is perhaps safe to say that in cases such as Patella (15), Limnæa (16) and Saccocirrus (7) the extrusions will be found to give a negative reaction with Feulgen's stain—a fact that is in accordance with the observations made by Ludford and Gatenby. Again, if the chromosome theory be true (as unquestionably it is) all the various basophil-staining bodies of germ-cells, hitherto described as chromatinic in nature, will be found to have a different source of origin, other than chromatinic. The occurrence of so-called basophilic extrusions in the epithelial cells of the epididymis of mammals is another example, where by the application of Feulgen's method Ludford failed to find any indication of chromatin being extruded into the cytoplasm.

Yet there are cases where certain structures have been found in the cytoplasm giving a positive reaction to the nucleal staining. Such for example are certain cells from cataract in a dog (Westhues, 22, quoted by Feulgen) and from carcinoma of a mouse (Roskin, 20). In both of these examples chromatin emission has been claimed to be established by means of the Feulgen reaction. On the other hand, an application of this method by Ludford (17) to certain tar tumours has proved that the nuclear extrusions in these cases consist only of nucleolar material.

It would appear doubtful whether true emission of chromatin does take place in normal tissue cells. How then are we to account for the examples given by Westhues and Roskin ? It appears that such extrusions may take place under abnormal conditions in diseased organs of the body, where the

nuclei of the cells, instead of functioning normally, undergo various processes of degeneration, throwing out chromatinic granules into the cytoplasm. This is evident from Roskin's own account of cells in which he finds the chromatin carried to one side of the nucleus. Evidently this is abnormal, and perhaps represents a condition to be followed subsequently by the emission of chromatinic granules. Certainly this is substantiated by the observation of Roskin himself, who found two types of nuclei—one with more chromatinic granules and the other with less. Probably something of the same type of changes take place in the cells of cataract of dog. The chromidia of Actinosphærium demand special attention, and until the chromatin test is applied in conjunction with other up-to-date cytological techniques, the chromatinic nature of these chromidia cannot be taken for granted.

The application of Feulgen's technique to the oogenesis of Apanteles fails to reveal any relationship between the secondary nuclei and the germ-cell determinants, and the chromatin of the nucleus. They give a negative reaction, indicating their origin from something other than chromatin. An objection may here be raised, that the germinal vesicle at the end of the prophase of heterotypic division, even though it contains the chromatin, likewise fails to give the nucleal reaction; but this can easily be explained when one examines such a stage stained in iron-hæmatoxylin. Even with this stain the chromatin material remains invisible. The inference that necessarily follows is that the nucleus, at this stage, contains chromatin in such a finely divided state that neither the Feulgen's reaction nor the iron-hæmatoxylin stain is able to demonstrate them under the highest power of the microscope.

There is another explanation mentioned by Koch (13), who states that during the growth of the oocyte the chromatin undergoes a profound chemical change. This chemical change, if it happens at all, must be of the nature of a reversible reaction, or otherwise the chromatin granules would not be reconstructed. A physical alteration is therefore quite probable, but until new investigations have been carried out, further discussion is not indicated.

Summary.

1. According to the Feulgen test the secondary nuclei in the oocytes of *Apanteles sp.* contain no chromatin, and do not originate as chromatinic emissions from the head-nucleus.

2. The germ-cell determinant is not of a chromatinic nature. It does not stain with the "nucleal reaction."

3. The basophilic constituents of the germ-cell determinants are not dissolved away by acid hydrolysis.

4. The germinal vesicle at the end of the prophases of heterotypic division gives a negative reaction to Feulgen's staining.

5. There is no emission of chromatin from the follicular cells into the oocytes or the mature eggs.

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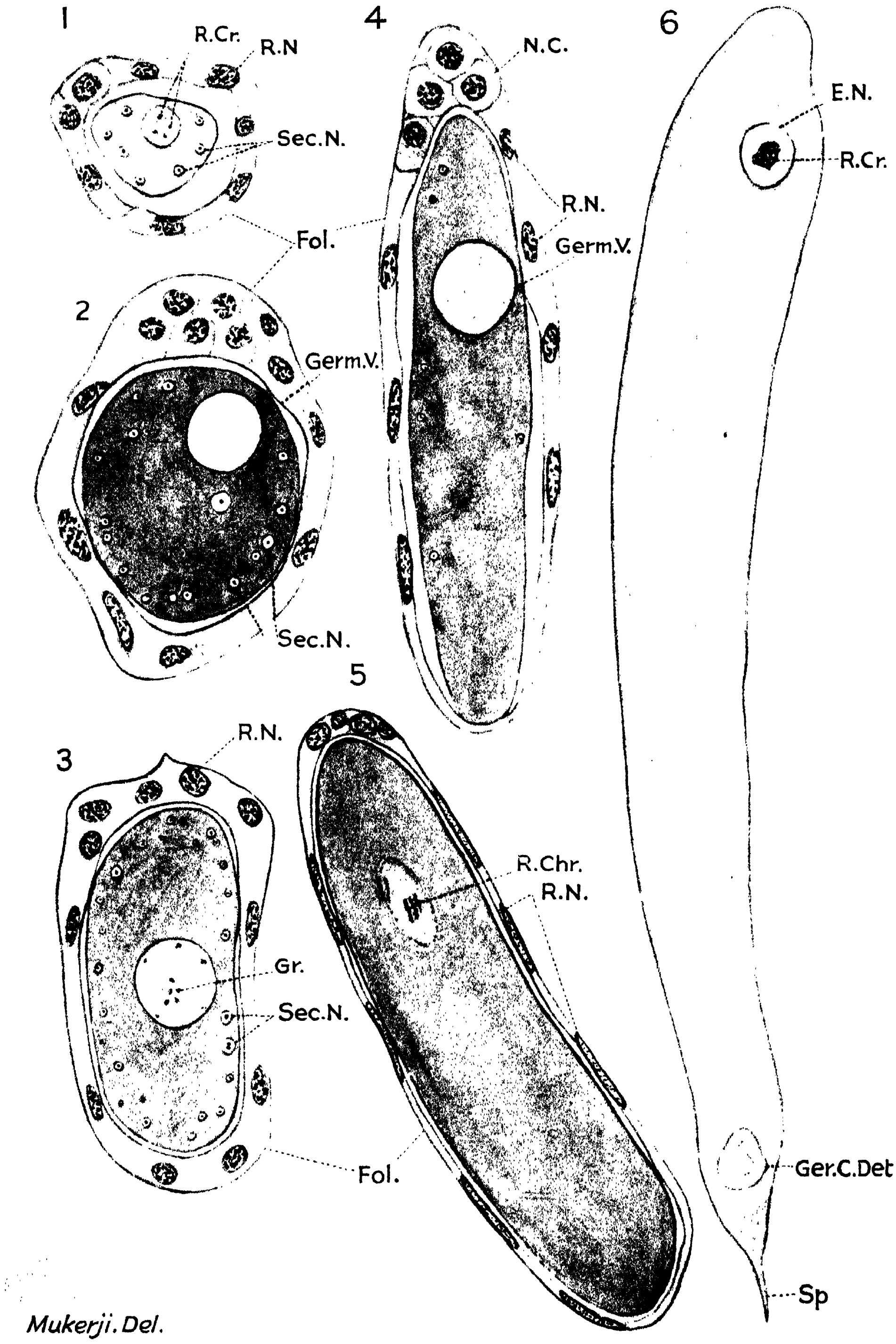
1
R.Cr.
R.N
Sec.N.
Fol.
2
Germ.V.
Sec.N.
3
R.N.
Gr.
Sec.N.
Fol.
4
N.C.
R.N.
Germ.V.
5
R.Chr.
R.N.
6
E.N.
R.Cr.
Ger.C.Det
Sp
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EXPLANATION OF PLATE 12.

Lettering of Figures.

E.N., egg-nucleus.
Fol., follicle.
Gr., granules (coloured green).
Germ. V., germinal vesicle.
Ger. C. Det., germ-cell determinant.
N.C., nurse-cell.
R.N., nuclei of follicular cells (coloured purple).
R.Cr., chromatin granules (coloured purple).
R.Chr., chromosomes (coloured purple).
Sec. N., secondary nuclei (coloured green).
Sp., spine.

Fig. 1.—A young oocyte. The secondary nuclei are coloured green. The chromatin granules are very few in number.

Figs. 2 and 3.—Fully-grown oocytes after the prophase of heterotypic division. No chromatin is visible. The germinal vesicle in fig. 3 with a few granules stained green.

Fig. 4.—A fully-grown oocyte; a stage slightly later than fig. 2. The secondary nuclei are very few in number.

Fig. 5.—An oocyte in the process of maturation. The chromosomes are coloured purple.

Fig. 6.—A ripe egg from the bottom end of the tubule: chromatin coloured deep purple, and the germ-cell determinant lightly green.

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Para-Crinkle: a Potato Disease of the Virus Group.

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(From the Potato Virus Research Institute, Cambridge.)

(Communicated by Dr. H. H. Dale, Sec.R.S.—Received December 18, 1929.)

[Plates 13-17.]

The investigations here recorded were prompted by the confirmation, early in 1928, of observations made by the senior author in 1927, that when apparently healthy King Edward plants were grafted on to the two test plants, President and Arran Victory, the latter succumbed but the former did not. This work, so far as it was directed toward the elucidation of this particular phenomenon and its relation to a new disease entity, was initiated by both authors and proceeded actively till March, 1929, when the junior author left to take up the post of Entomologist in Kenya. Since then the senior author has continued the study and is alone responsible for the presentation of the work and the deductions as they now appear.

Introduction.

In June, 1927, the senior author, in company with Prof. Paul A. Murphy, of Dublin University, and Mr. W. D. Davidson, Inspector of Irish Free State Ministry of Agriculture, visited various parts of Athlone and Donegal on a tour of potato inspection, and selected from several localities plants of different varieties which appeared to them to be without sign of disease. From such plants, immature tubers were removed, placed in paper bags and brought back to the Research Station at Cambridge, and kept under insect-proof conditions. In 1928 each tuber was divided, and one half grown-on in the insect-proof glasshouse, the other in the open.

This paper is concerned only with the behaviour of King Edward amongst this Irish selection. There were four units, *i.e.*, four tubers from four individual plants, of Irish King Edwards, tested in 1928, and below is a record of their behaviour :—

(1) (Innishowen). In the glasshouse; a strong plant, but the leaves not of a perfectly normal colour and texture. A shadowy and hardly perceptible mottling observed on some leaflets.

Grafted to healthy President H.P./1875 = stock developed mosaic.

Grafted to Healthy Arran Victory H.A.V./2017 = stock developed mosaic.

(2) (Gore-Booth). In the glasshouse--strong and healthy.

Grafted to Healthy President H.P./1865 = stock developed mosaic.

Grafted to Healthy Arran Victory H.A.V./1232 = stock developed mosaic.

(3) (Innishowen). In the glasshouse—a strong plant but the foliage was so suggestive of mottling that the unit was discarded without further testing.

(4) (Innishowen). In the glasshouse—strong plant apparently in perfect health.

Grafted to Healthy President H.P./1660 = stock remained healthy.

Grafted to Healthy Arran Victory H.A.V./2019 = stock developed severe crinkle.

Grafted to Healthy Arran Victory H.A.V./2219 = stock developed severe crinkle.

The behaviour of these four units under test, whilst confirming previous observations on material which had not been especially selected and which had not been so carefully examined whilst growing, presented a problem which was both new and intriguing. In the first place, of four plants selected under the auspices of three persons who, whatever else their qualifications, might be credited with recognising a healthy plant when they saw one, none had survived the more critical tests of the following year. Three of the units were condemned on account of mosaic disease, which, in the case of the two tested by grafting, was much more apparent in the test plants than in the King Edward plant under trial; the fourth plant was evidently harbouring some very distinctive disease whilst itself retaining the full bloom of health and vigour.

The idea of the "carrier" is not new in the literature of plant virus diseases. Murphy (5) had described the condition in several varieties. The senior author had met it in such varieties as Irish Cobbler, Uptodate, and in the wild species *S. utile*; but these carriers would not have been prize winners at a health show, as would our King Edward carriers, nor would they have escaped

a rigorous examination without disclosing some tell-tale stigma suggesting the presence of virus disease.

It should be mentioned that the development of " Crinkle " in Healthy Arran Victory after grafting on to it an apparently healthy King Edward, had been noted in 1927, but its significance was not realised till the succeeding season. In the same manner, the importance of the fact that in our series of 1927 grafts double as many scions succeeded in producing one virus disease or another on Arran Victory as they did on President, escaped immediate attention. In 1928 grafting was carried out more systematically and under far better conditions. Every plant to be tested was grafted on to both Arran Victory and President; the Victory plants showed lesions in 72 per cent. of cases, the President in 62 per cent. The explanation of this difference lies in the fact that whenever Para-Crinkle* occurred in the tested plant, Arran Victory recorded it, whilst President remained unresponsive.

Disquieting, however, as was the discovery of the really perfect carrier, it was still more so to realise that one and the same disease, which when brought by grafting into Arran Victory induced a severe disease in it, when introduced by the same method into President failed to produce the slightest reaction. Here indeed was, and is, a threat to the validity of much of the work which has been done on virus diseases of the Potato. That our observation gives the quietus to the method of testing for the presence of virus diseases in the Potato by inspection of the host plant only, whether under glass or in the field, will disturb most workers as little as it did the authors; but that the variety President, which has been the mainstay of our and others' tests for the presence or absence of mosaic diseases in general, should completely fail to react to a classical and severe example of what hitherto had been regarded as a typical virus disease, was sufficiently disconcerting to induce us to concentrate on the problem it presented.

The basis of any exact study on the virus diseases of the Potato must be the possession of controls that are free from such diseases. It is not the least of the debts which workers owe to Prof. P. Murphy, of Dublin, that he has isolated, and maintained, stocks of two varieties, viz., President and Arran Victory, in a complete state of health for several years, and that he has been generous enough to give co-workers tubers of his stocks. Our work has based itself on this secure foundation. It has been stated above that President can " carry " a very severe disease, which we shall call Para-crinkle, without

* It is proposed that the form of Crinkle which is evident in Arran Victory but latent in President, should be given the name of Para-crinkle.

showing the slightest sign of distress ; it might therefore be suspected that our control stocks of President are not necessarily healthy, but might themselves be carriers. Arran Victory is a variety which we have found to be peculiarly susceptible to Para-crinkle, and we have frequently grafted our healthy President control units on to Arran Victory without producing in the latter any symptoms ; nor, conversely, have we produced any disease symptoms by grafting Arran Victory on to President. If we graft either our Arran Victory or President on to a third stock, we never have, by so doing, initiated symptoms of disease on the latter ; or where the latter was itself diseased, increased or modified by such grafting the symptoms already existing.

In view of the repeated statements of Johnson (3 and 4) in which he claims to have produced virus diseases of greater or lesser virulence in the Tobacco by inoculating the same with juices of healthy Potatoes, it seemed of importance to clear the ground by testing-out our standard controls, and to make preliminary experiments with recognised Potato-virus diseases by inoculating their juices into Tobacco and other Solanums. The result of this examination is seen below :—

Table I.

Variety.	Unit.	Juice from Tuber.	Juice from Sprout.	No. of Tobacco inoculated.	No. of Tobacco reacting.	No. of Tomato inoculated.	No. of Tomato reacting.
President ..	10A	+		9	Nil	9	Nil
,, ..	10A		+	9	Nil	9	Nil
,, ..	10.1A		+	17	Nil	—	—
,, ..	4		+	5	Nil	5	Nil
,, ..	7B and 7C (Mixed juice)		+	4	Nil	—	—
				44	Nil	23	Nil

We have made 44 separate inoculations of healthy President into Tobacco, and 23 into Tomato, without producing any pathological effects ; our colleague, Dr. Kenneth Smith, has made similar inoculations with President on 20 Tobaccos, with completely negative results.

With healthy Arran Victory 62 inoculations (Table II) were made into Tobaccos with negative results ; to these may be added 20 similar inoculations made by Dr. Kenneth Smith.

In four further inoculations the juices of healthy Arran Victory and healthy President were mixed and inoculated into Tobaccos with no result.

Table II.

Variety.	Unit.	Juice from Tuber.	Juice from Sprout.	No. of Tobacco inoculated.	No. of Tobacco reacting.	No. of Tomato inoculated.	No. of Tomato reacting.
Arran Victory	4A	+		8	Nil		—
,, ,,	4A		+	8	Nil	8	Nil
,, ,,	2A	+		23	Nil		—
,, ,, ...	2A		+	23	Nil	7	Nil
				62	Nil	15	Nil

In all, we have made at Cambridge 146 inoculations of the juices of our two healthy stocks, Arran Victory and President, into Tobaccos, without producing a single lesion.

Further tests were made of our two control healthy plants as follows :—

Healthy President—6 plants inoculated into Datura. No symptoms.
Healthy Arran Victory—2 plants inoculated into Datura. No symptoms.
Healthy President—2 plants grafted into Datura. No symptoms.
Healthy Arran Victory—1 plant grafted into Datura. No symptoms.

In addition, 22 inoculations were made of the healthy stocks into Hyoscyamus, 5 into Petunia, 25 into *S. melongena*, with equally negative results. Henderson Smith (8) obtained similarly negative results following inoculation into Tomatoes of nine different healthy Potato varieties.

The complete testing of our two stock test plants with uniformly negative results, has given us confidence that in them we have reliable tools to work with.

It is not our intention to controvert Johnson's findings further than to say that, when strictly healthy plants are used, no symptoms are produced in Tobacco (or other Solanums) by injecting into the latter the juice of the Potato ; nor do we find any evidence for the existence of a virus as the normal constituent of a normal Potato plant. We shall, however, have occasion to point out how readily certain of the mosaic group of virus diseases may lie hidden in apparently healthy Potatoes.

The literature which has grown up around the so-called virus diseases of the Potato has, to an increasing extent, concerned itself with the attempt to demonstrate the specificity of certain forms of disease. The question as to whether these diseases are strictly virus diseases—*i.e.*, brought about by the presence in the tissue of the host plant of a filtrable virus—has fallen into the

background; nor is the reason far to seek, for unless the filtered juice can by artificial inoculation be made to reproduce the disease (and inoculation into the Potato is by no means generally successful) proof is lacking.

The diseases which are commonly described as virus diseases may be placed into three groups: Leaf Roll, Mosaic and Streak. In regard to the first, there is no evidence of successful infection by artificial inoculation of the plant juice. In the second group, containing as it does several clinically distinct forms, the evidence lacks uniformity inasmuch as some forms have been so transferred and others have not. The third group is again a complex one, and it is only in a few cases that the symptoms can be made to appear in healthy plants by inoculation methods. Very little work has so far been done on the filtered juices of diseased Potato plants, and next to none on the properties of the virus *in vitro*. Notwithstanding the difficulties referred to, workers have not felt themselves debarred from researching into these groups of Potato diseases on the basis of their being true virus diseases. We are of opinion that the striking analogies of the diseases in question to the proved virus diseases of such plants as the Tobacco, the Tomato and the Cucumber, are a sufficient justification.

The work of our colleague, Dr. Kenneth Smith (9), on the Ring-spot Mosaic of Tobacco, induced by inoculation of healthy Tobaccos with mosaic Potato juice, and the infectivity of the filtered Tobacco juice both to Tobacco and Potato, is strong evidence of the presence of a similar filtrable virus in the mosaic Potato plant itself. Indeed, Smith finds that the juice of a Potato plant suffering from Ring-spot Mosaic, *i.e.*, the disease induced in the Potato by infecting it with the juice of a ring-spot Tobacco, when filtered successively through Berkfelt filters L2 and L3, was capable of infecting by inoculation both healthy Potatoes and Tobacco. Unless the presence of the Tobacco plant can be held to invalidate the argument, the chain of evidence for the filtrable-virus character of the Potato mosaic disease is complete. Henderson Smith (8) has filtered the juice derived from Potato mosaic plants of several varieties and by inoculating Tomatoes with the filtrate has demonstrated their infectivity.

Recently Johnson (3) has found that Rugose Mosaic of the Potato, which is allied to, if not identical with, a form of Crinkle we have called Crinkle "A"* and which is clinically like Para-crinkle, cannot be reproduced from the filtered juice. Even if this result is confirmed by other workers, one would yet hesitate to exclude Rugose Mosaic from the virus group, so strictly com-

* The subject of Crinkle "A" is dealt with fully in a previous communication (6).

parable is it in its clinical and other characters to the diseases which have been found to be due to filterable viruses.

The equipment of the Potato Research Institute at Cambridge does not at present allow of the investigation of virus properties *in vitro* ; hence we cannot deal with that aspect of the question, though it is hoped that we shall be in a position to do so before long.

Putting, therefore, to one side the question as to whether the various mosaic diseases of the Potato are, in the strictest sense, virus diseases or not, we may pass on to the question of the specificity of the diseases so described. And here we are met with a two-fold difficulty : one temporary and relatively unimportant—that of nomenclature ; the second very real and vital—that of varietal reaction to one and the same infective source. It is not the purpose of this paper, nor indeed is the time yet ripe, to deal effectively with the former, but the latter, important though it is in some of the other Potato virus diseases, is peculiarly so in respect to the particular one with which we are concerned. Difference in reaction between one variety and another to the stimulus of a single pathogen is responsible for some at least of the confusion in nomenclature, and for bringing about the unnecessary elevation of a particular varietal symptom-complex to the dignity of a specific disease. We shall find, however, in this connection, the reverse phenomenon, for it is varietal inaction to a certain unit pathogen which has led to the splitting up of what hitherto has been one of the best defined symptom complexes, viz., "Crinkle," into at least two quite distinct diseases, obviously induced by distinct pathogens.

The outstanding difficulty in the study of plant virus diseases is the fact that we have in so many cases to rely on the clinical picture alone for the identification of the pathogen. In a few cases, it is true, the active pathogen may be to some extent isolated and extracted from filtered plant juice, as Vinson and Petre (11) have done for Tobacco, and Brewer and his colleagues (1) for Tomato ; but in no case has it been cultivated *in vitro*, and in no plant has any reaction of immunity been discovered which could indirectly lead to the identification of a specific virus.

The clinical picture of Potato virus diseases may vary with the variety of the plant, even when the difference is morphologically so unimportant as it is, say, between two Potato varieties, such as Great Scot and Arran Chief. There is, however, the further complication which may arise from the variable reaction of the diseased plant, as exhibited by its clinical appearance, to variations in temperature, light and soil. Indeed we have good reason to believe that such relatively small changes in environment as may exist between

the soil and climatic conditions of culture in certain parts of Scotland and that in the south of England determine the gross differences that commonly ensue when a virus-infected stock is grown in the two districts. This, however, opens up the consideration of a difficult and important problem, viz., the relation of the health of the crop both to the internal, *i.e.*, the physiological and pathological, conditions of the seed tuber from which it springs, and to the external or environmental conditions. This problem can now be hopefully attacked, if only we are justified in believing that the stocks of the varieties used in our experiments are really virus free.

It is considerations of this nature which have given both point and stimulus to the work of the Virus Research Institute at Cambridge, in its endeavour to secure, raise and maintain virus-free stocks of the more important commercial varieties. It is not proposed to enlarge here on the methods employed further than to say that repeated tests, by inspection, grafts and inoculations, are made before any one plant is accepted as free from disease, and that both during and after this examination, which continues over several years, the stock is maintained in an insect-proof glasshouse. We have by such methods obtained small stocks of varieties which we believe to be sound. We are not equally confident as to all, but at least it can be said of all the plants used in this research that the units from which they spring have been repeatedly tested and are, as far as we know, free of any virus disease. Our experience, and especially that of the senior author (6) as detailed in the communication on Crinkle "A," teaches us that a very mild mosaic in the varieties Abundance and Kerr's Pink may itself be a varietal modification of a much more serious disease which may be almost suppressed, and in such manner lead to infected stocks of these varieties passing as virus-free.

There is evidence that stocks which are infected with a virus of which they themselves have reduced the immediate virulence, or are infected with strains of a virus attenuated by passage through other varieties, are rendered particularly susceptible to change of environment. Evidence is accumulating both in volume and worth which tends to show that such virus-free stocks as have been worked up at Cambridge are in a sense physiologically independent of the environment. They are frozen by the cold or flag in the heat like any other, but they are not in the least affected by the locality of the seed's origin, nor by the length of years during which they have flourished south of the border, nor do they appear to react to unfavourable soils and the like as do ordinary stocks. The elasticity of environmental response, which was lost by disease, has been restored.

Table

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
King Edward stocks.					Grafts in 1927.			
Source and unit.	Date.	Field condition in.	Pot No. tested.	Condition of plant in pot.	Arran Victory.	Field plot, 1928.	President.	Field plot, 1928.
Scotch—								
R. 1	1926	Mild mosaic	—	—	—	—	—	—
R. 1	1927	Suspicious	—	—	—	—	—	—
R. 2	1926	H	158	H	H	H	H	? H
R. 2	1927	Mild mosaic	358	H	Crinkle ...	Curly Dwarf	H	—
R. 2	1928	H	1369	H	—	—	—	
R. 2	,,	—	424	H	—	—	—	—
R. 2	,,	—	425	H	—	—	—	—
R. 2	,,	—	427	H	—	—	—	—
R. 3	1926	Suspicious	160	H	H	—	H	H
R. 3	1927	H	161	H	H	—	H	H
R. 3	1928	H	1379	H	—	—	—	—
R. 3	1929	H	433	H	—	—	—	—
R. 3	—	—	435	H	—	—	—	—
R. 4	1926	H	164	H	Very suspicious	Crinkle	H	H
R. 4	1927	H	1385	H	—	—	—	—
R. 4	1928	Suspicious	438	H	—	—	—	—
R. 4	—	—	440	H	—	—	—	—
R. 5	1926	Suspicious	165	H	Crinkle	Curly dwarf	H	Suspicious
R. 5	1927	H	2416	H	—	—	—	—
R. 5	1928	H	314	H	—	—	—	—
R. 5	—	H	314	H	—	—	—	—
R. 5	—	H	1528	H	—	—	—	—
R 5.	—	H	314	H	—	—	—	—
R. 5	—	H	314	H	—	—	—	—
R. 5	—	H	314	H	—	—	—	—
R. 5	—	H	314	H	—	—	—	—
R. 6	1926	H	167	V. mild mosaic	Crinkle	Curly dwarf	H	Suspicious
R. 6	1927	H	—	—	—	—	—	—
R. 7	1926	H	—	—	—	—	—	—
R. 7	1927	H	—	—	—	—	—	—
R. 7	1928	? LR	1631	H	—	—	—	—
R. 8	1927	H	—	—	—	—	—	—
R. 8	1928	H	1632	H	—	—	—	—

H = Healthy. L.R. = Leaf Roll.

II.

(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)
Grafts in 1928.				Grafts in 1929.		Additional pots of units grown for observation.	
Arran Victory.	Field plot, 1929.	President.	Field plot, 1929.	Arran Victory.	President.	Healthy.	Diseased.
—	—	—	—	—	—	0	0
—	—	—	—	—	—		
—	—	—	—	—	—	30	0
—	—	—	—	—	—		
Scion did not grow. Very suspicious	Curly dwarf	H	H	—	—		
—	—	—	—	Crinkle	H		
—	—	—	—	Crinkle	H		
—	—	—	—	Crinkle	H		
—	—	—	—	—	—	27	0
—	—	—	—	—	—		
Suspicious	Curly dwarf	H	H	—			
—	—	—	—	Crinkle	H		
—	—	—	—	Crinkle	H		
—	—	—	—	—	—	24	0
H	Suspicious	H	H	—	—		
—	—	—	—	Crinkle	H		
—	—	—	—	Crinkle	H		
—	—	—	—	—	—	—	—
Crinkle	—	H	—	—	—		
				314 \			
—	—	—	—	Crinkle/227	—		
—			—	Crinkle/8	H		
—	—	—	—	Crinkle/247	H		
—	—	—	—	Crinkle/5	H		
—	—	—	—	Crinkle/228	H		
—	—	—	—	Crinkle/499	H		
—	—	—	—	Crinkle/229	H		
—	Curly dwarf from 1927	—	Strong plant V. faint mosaic from 1927	—	—	17	2
—	—	—	—	—	—		
—	—	—	—	—	—	1	0
—	—	—	—	—	—		
—	—	Mosaic	—	—	—		
—	—	—	—	—	—	1	0
Mosaic and LR.	Curly dwarf	H	H	—	—		

Table III.—

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
King Edward stocks.					Grafts in 1927.			
Source and unit.	Date.	Field condition in.	Pot No. tested.	Condition of plant in pot.	Arran Victory.	Field plot, 1928.	President.	Field plot, 1928.
Scotch—								
R. 9	1927	H	730	H	—	—	—	—
R. 9	1928	H	1633	? H	—	—	—	—
R. 10	1927	Very poor	731	? H	—	—	—	—
R. 11	1927	Mosaic	732	? H	—	—	—	—
R. 12	1927	Suspicious	1634	H	—	—	—	—
R. 13	1927	Mosaic	734	? Mosaic	—	—	—	—
R. 14	1927	H	735	? H	—	—	—	—
Irish—								
I. 1	1927	H	1728	H	—	—	—	—
I. 2	1927	H	1729	? H	—	—	—	—
I. 2	1928	Mild mosaic	—	—	—	—	—	—
I. 3	1927	H	1730	H	—	—	—	—
I. 3	1928	H	1730	H	—	—	—	—
G.B. 1	1927	H	1731	? H	—	—	—	—
Cumberland	1929	H	Fired	only	—	—	—	—

H = Healthy. L.R. = Leaf Roll.

We have alluded to this matter of virus-free stocks here for the purpose of emphasising the overwhelming importance of virus infection in the Potato. It will be seen that the disease of Para-crinkle may be latent to a degree unsuspected of virus diseases before; and further, that it, like Crinkle "A," may be masquerading under the guise of so slight a mosaic affection as to be scarcely noticeable. It will, however, be shown that the virus which lurks unseen in one variety may, by transference to another, reduce it to impotence.

The difficulty which exists in securing virus-free stocks of commercial varieties has prompted the suggestion that for research purposes seedlings might be used, for the evidence that they are congenitally free from disease is very strong. However, this line of advance is also blocked, for as the senior

(continued).

(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)
Grafts in 1928.				Grafts in 1929.		Additional pots of units grown for observation.	
Arran Victory.	Field plot, 1929.	President.	Field plot, 1929.	Arran Victory.	President.	Healthy.	Diseased.
— —	— —	— —	— —	— —	— —	— —	— —
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
Crinkle	Curly dwarf	H	H	—	—	—	—
—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—
? Mosaic	—	Mosaic	—	—	—	—	—
— —	— —	— —	— —	— —	— —	} 2	0
Crinkle Crinkle	Curly dwarf Curly dwarf	H —	H —	— —	— —	— —	— —
Mosaic	—	Mosaic	—	—	—	—	—
—	—	—	—	Crinkle	—	—	—

author (7) has shown, Potato varieties are in general heterozygous for most of their visible characters, and to no less degree for such physiological ones as can be determined, such as maturity and susceptibility to Wart Disease and Phytophthora. Seedlings of any given variety or any given cross would not be uniform material; indeed their use would introduce, with every plant employed, a special varietal reaction—an impossible state of affairs for virus-disease research.

Our experimental work has been conducted on material the nature of which has already been alluded to, and has been conducted in special insect-proof glasshouses. The temperature of the houses from April to September has ranged round an average of 78·6° F. during the day, and 51° at night. It has

been found essential, in order to demonstrate symptoms, to employ actively-growing plants, and trials have shown that neither in the late winter nor the late autumn months can reliable results be obtained; the plants, notwithstanding artificial light and heat, are not in a sufficiently active state of metabolism to react to the virus within them.

As further aids in the work, we have made a series of trials with Tobaccos, Tomatoes, Hyoscyamus, Petunia, Capsicastrum, *S. esculenta*, Nicandra and Datura. To all of these there are objections: either they do not react at all to some of the viruses employed, or their reaction is uncertain or transient. Of all, Datura has proved the most efficient; for it does clearly differentiate between Crinkle "A" and Para-crinkle, and gives a constant and uniform reaction towards the former. Tobaccos are of value also, but not to the same extent, and here, moreover, there are very considerable differences of reaction between different varieties of Tobacco, and in addition individual differences of reaction within the same variety, as Kenneth Smith (10) has shown. In our research on Para-crinkle, we have made use of both Datura and Tobacco, but it is the former only which has been of material assistance.

We may now turn to the evidence for the existence and behaviour of this new disease, which we have ventured to call Para-crinkle.

Table III contains evidence as to the condition of the 19 various stocks of King Edward examined. In column 1 are the tuber units tested, viz., 14 from Scotland, 4 from Ireland, and 1 from Cumberland. Some units have been tested many times, thus R. 5, it will be seen, has been grafted to the test plants on 9 different occasions. Column 2 gives the dates under which the stocks were first examined in the field or house, and column 3 the condition of the field plots as judged by inspection only. Column 4 records the pot number of the actual plant of any given unit examined and column 5 the condition of the stock plant as grown in the glasshouse. Columns 6 to 9 give the result of the tests in 1927 on the pot plants of Arran Victory and President respectively, and also the appearance of the products of these pots in the succeeding year when planted out in the field. Columns 10 to 13 give the same information for those units examined in 1928, and columns 14 and 15 that for the pot test plants of 1929. Column 16 records the number of additional pots of stock plants of King Edward grown of each unit which were healthy on inspection, and column 17 those which showed any evidence of virus disease. Fig. 1, Plate 13, is a photograph of one of these "healthy" King Edward plants.

In all, 19 units were examined: of these 7, viz., R. 1, R. 9, R. 10, R. 11, R. 13, R. 14 and I. 1, were turned down on inspection only as being either obviously

affected with mosaic disease or sufficiently varying from a condition of perfect health as to be regarded with the gravest suspicion.

Of the 12 remaining units, 8 when grafted to Arran Victory produced in the latter on at least one occasion in each unit a definite crinkle ; of the 3 remaining, 1, viz., R. 8, although it gave, in the single test applied, a mixture of leaf roll and mosaic, must be added to the first group, because when the Arran Victory test plant was grown on in the following year it developed into curly dwarf, which we shall see is characteristic of Para-crinkle. This leaves 2 units, viz., R. 7 and I. 1, which reacted to Arran Victory by producing on the latter a simple mosaic, whilst in each case the President test plant reacted by also developing a simple mosaic.

Leaving then the last 2 units for the moment out of consideration, and returning to the remaining 8, we find that they were grafted to healthy Arran Victory and healthy President with the following results :—

Table IV.

Number of King Edward units tested.	Healthy Arran Victory test plants.			Healthy President test plants.	
	Crinkle.	Other virus diseases.	Healthy.	Diseased.	Healthy.
8	21	1 Mosaic and Leaf Roll; 3 suspected of Mosaic	4	0	24

If we look more closely into the occurrence of disease in the Arran Victory test plants, we find that, in 1927, seven grafts were made with the result : 3 Crinkle, 1 suspected of Mosaic, 3 Healthy ; in 1928 seven grafts were made, resulting in : 4 Crinkle, 2 suspected Mosaic, 1 Healthy ; and in 1929 thirteen grafts were made and all developed Crinkle.

It is clear, seeing that the units examined are more or less common to all the three years, that we have another factor involved which has brought about a fuller and more prompt reaction. This factor in a word is "competence." In 1926 the senior author tried out several methods of grafting and after-care, but had to record a very large percentage of failure ; in 1927 we had just moved into our new insect-proof houses and were still trying out methods ; in 1928 we made considerable progress in the matter of the after-treatment for grafted plants ; whilst in 1929 the senior author devised special box lights within the houses which, with suitable care, allow us now to obtain about 97 per cent. of successes with all our grafts. We are of opinion that had we been in

possession of the procedure finally developed, in 1927, we should have found that the whole of our Arran Victory test plants would have displayed Crinkle on being grafted with any of the units under consideration. The reaction in the President is, however, uniform: 24 plants grafted, and all unaffected.

Reverting to the 8 King Edward units which have given a crinkle in Arran Victory, and no reaction in President, we see from Table V. that these units when examined in the field and in the glasshouse behaved as below:—

Table V.

Unit.	No. of times tested in field.	Healthy in field.	Disease in field.	No. of pots grown in glasshouse.	Healthy in glasshouse.	Diseased in glasshouse.
R. 2	3	3	0	36	36	0
R. 3	4	3	1 suspicious	32	32	0
R. 4	3	2	1 ,,	28	28	0
R. 5	2	1	1 ,,	8	8	0
R. 6	2	1	1 ,,	18	18	0
R. 8	2	2	0	1	1	0
R. 12	1	0	1 suspicious	0	0	0
I. 3	2	2	0	2	2	0
Cumberland	1	0	1 Mild Mosaic	0	0	0
	20	14	6	125	125	0

From this it appears that as a result of inspection in about 25 per cent. of the field plots some trouble may be suspected, yet it is insufficient in degree even to be diagnosed as a mild mosaic; inspection, however, of pot plants in the glasshouse fails to reveal the presence—or even excite the suspicion of such—of any disease whatever in this variety. We are therefore able to say that Para-crinkle in King Edward cannot be detected by inspection either in the field or in the glasshouse, and that only by grafting on to the variety Arran Victory (we shall see there are other tell-tale varieties) can its presence be determined.

The facts recorded in Tables IV and V throw some light on the question of the behaviour of "carriers." King Edward is a carrier, but is clearly not a consistent one under all conditions. In the first place we see that the environment of the hot-house converts King Edward into a perfect carrier of Para-crinkle, and that of the outside world into a less consistently perfect one. Moreover, in the case of practically every unit, plants of the same source differ from year to year in the degree with which they hide the symptoms.

It appears that the uniform condition of high humidity and temperature

of the glasshouse helps to maintain the healthy appearance of the carrier King Edward, and that as the conditions prevalent in the field differ in different years, so the expression of symptoms tends to vary also. Variable, however, as the symptoms are, they are but a feeble indication of the virulent disease the variety is harbouring.

The variety President is also a carrier of Para-crinkle; of the 27 pot plants inoculated with King Edward carrying Para-crinkle all remained healthy.

In Table VI are detailed those President plants which have been infected with Para-crinkle by grafting from King Edward or other sources, and have either been grown on in the succeeding year in field plots and/or grafted to Arran Victory to test whether they are harbouring the virus.

Sixteen Presidents were grown on in field plots in the succeeding year; one of them also in a second year. In all 17 plots the plants were vigorous, in 1 mosaic disease was suspected, and in 4 plots including the one tested over two years, the growth though vigorous was regarded with suspicion; no definite diagnosis of mosaic disease was, however, made. One such President plant was regrafted to Arran Victory, which developed Para-crinkle in consequence.

Six more of the infected but healthy-looking President plants were grafted to Arran Victory, one of them twice. Five of them induced Para-crinkle in the Victorys and in one case, Pot 230, the Arran Victory was regarded with suspicion only. It is of interest to note the history of the President in this case: it was grafted with a very small scion of King Edward, 11.2.29; this scion never grew, but held its own till 11.3.29, when it began to wilt and died by the end of the month. It is clear that the President was never efficiently infected.

In fig. 2 is seen a very vigorously-growing President scion, carrying Para-crinkle, with which it has infected the Arran Victory stock.

It is obvious that President will convey the virus of Para-crinkle in a latent condition, whether it obtains the same from sources such as King Edward or Abundance, where it itself is latent, or from Arran Victory, where it is fully expressed clinically. Other infections of President, not recorded in the table, were made from sources such as Para-crinkle infected Arran Chief, with a similar result, viz., no apparent injury to the health of the plant.

In only one case did President show any reaction after grafting with Para-crinkle, and that was where the source, an infected Arran Victory, had been grown on for three seasons, and when used as a scion to a healthy President induced in the latter a mild but definite mosaic. (Pot 734P.)

Table VI.—The Behaviour of the Variety President when infected with Para-Crinkle.

Year.	Source of infected scion.	Pot No.	Condition of President.	Pot No. of President.	President in field.	Field Plot No.	Regraft to Arran Victory.	
							Symptoms.	Pot No.
1927	King Edward 6 B	167	Healthy (1927)	229	Vigorous but suspicious	146/28	—	—
1928	King Edward 6 B	167	Healthy (1928)	1529	Vigorous but suspicious	277P/29	—	—
1927	King Edward 5 B	165	Healthy	250	Vigorous, ? mild mosaic	143/28	—	—
1927	King Edward 2 B	158	Healthy	394	Vigorous but suspicious	183/28	—	—
1927	King Edward 3 B	160	Healthy	896	Vigorous—healthy	185/28	—	—
1927	King Edward 4 B	164	Healthy	985	Vigorous—healthy	187/28	—	—
1928	King Edward 2 B	1369	Healthy	1653	Vigorous—healthy	56P/29	—	—
1928	King Edward 3 B	1379	Suspicious	1652	Vigorous—healthy	57P/29	—	—
1928	King Edward 4 B	1385	Healthy	1656	Vigorous—healthy	58P/29	—	—
1928	King Edward I 3A	1730	Healthy	1660	Vigorous but suspicious	262P/29	Para-crinkle	2266
1929	King Edward 5 B	314	Healthy	9	—	—	Para-crinkle	$9A_2$
1929	King Edward 5 B	314	Healthy	233	—	—	Para-crinkle Para-crinkle	233A $233A_2$
1929	King Edward 5 B	314	Healthy	230	—	—	Suspicious	230A
1929	King Edward 2 B	424	Healthy	424P	—	—	Para-crinkle	424PA
1929	King Edward 5 B	314	Healthy	5	—	—	Para-crinkle	5A
1928	Abundance (infected by King Edward)	2157	—	1846	Vigorous and healthy	265P/29	—	—
1928	Arran Chief (infected by King Edward)	2166	Healthy	693	Vigorous and healthy	271P/29	—	—
1928	Arran Victory (infected by King Edward)	2249	Healthy	1668	Vigorous and healthy	259P/29	—	—
1928	Arran Victory (infected by King Edward)	2019	Healthy	2215	Vigorous and healthy	256P/29	—	—
1928	Arran Victory (infected by King Edward)	1530	Healthy	2302	Vigorous and healthy	278P/29	—	—
1928	Arran Victory (infected by King Edward)	2284	Healthy	2285	Vigorous and healthy	279P/29	—	—
1928	Arran Victory (infected by King Edward)	2282	Healthy	2286	Vigorous and healthy	280P/29	—	—
1929	Arran Victory (infected by King Edward)	734	Mosaic	734P	—	—	Para-crinkle	734PA

Scions from the same source were subsequently grafted to five more healthy Presidents, and none of these showed any reaction. The President plant that did react was grafted to Arran Victory, and produced in the latter a definite attack of Para-crinkle ; grafted to another President, it produced a very mild mosaic ; grafted to Datura it produced a reaction which we shall see is characteristic for Para-crinkle ; but inoculated to Datura it produced a reaction more like that of Crinkle "A."

The question arises whether the exceptional behaviour of this one graft to President is due to the fact that the Para-crinkle source was several seasons old, or to some previous but latent infection of another virus in that particular President plant. Inasmuch as the five other President grafts—all of different units from 734P—displayed no reaction, it is possible that the latter explanation is the right one. The immediate control to 734P is under examination. Evidence for the increased virulence of the source 734 will be met later.

The appearance of Para-crinkle in Arran Victory.

Arran Victory was grafted with Para-crinkle carrying units of King Edward 31 times. Two grafts failed, in four cases the scion lived but produced no effect, in three more cases the Arran Victory plants were sickly but not definitely diseased, and in 22 a violent crinkle resulted.

The clinical symptoms develop very irregularly. The disease may sweep over the plant involving in the course of 24–48 hours every leaf of the upper and younger parts of the plant, fig. 3 ; when this happens, the first symptom is a very definite blotching with yellow spots, followed rapidly by deformity and waving. If the symptoms develop more slowly, the first sign is a single bright blotchy patch of chlorosis on the junction of the larger veins (fig. 8), but even at the earliest stage this is accompanied by local puckering and deformity allied with an early waving of the leaf edge (fig. 3). This latter feature is always very characteristic of Para-crinkle in Arran Victory (fig. 4, Plate 14). Clearing of veins is not so prominent nor so constantly an early feature as in Crinkle "A," but it occurs generally, and may be very marked (fig. 5). The blotchy spots soon increase in number and rapidly become thinned and partially necrotic, bringing severe deformities in their train (fig. 5). Growth of the leaflet is more or less completely arrested, and by reason of the waving, deformity and rugosity, they appear smaller than their actual area warrants (fig. 6). Necrotic spots are common on the surface of the leaf and short brown streaks on the veins of the under surface, as well as on the petioles and stems. In the first season the disease, the symptoms of which are chiefly displayed on

the younger upper growth, has no very serious effect on the stature of the plant; very different is it when the plant is raised in a subsequent season from diseased tubers (fig. 7, Plate 15). The stems are numerous, short and brittle; the leafage is clumped and grossly deformed, and all the lesions are intensified, but are still clearly, as regards their character and range, those of Para-crinkle as observed in the first season.

Perhaps the most interesting feature of Para-crinkle in Arran Victory is the appearance of plants derived from diseased tubers when grown in the field. The plants are dwarfed, often not more than 6 or 9 inches high; the entire plant (fig. 9) presents the typical picture of Curly Dwarf; the leaves are clumped and twisted, and distorted almost beyond recognition, so that such plants look more like cabbages than potatoes.

The curly dwarf condition is sometimes incomplete: one part of the plant may exhibit extreme crinkle and another curly dwarf. However, it has been our constant experience (see Table III) that any plant of Arran Victory (and Arran Chief) which exhibits crinkle in the hot-house, develops in the succeeding year when grown outside into typical curly dwarf.

Curly dwarf has been regarded as a separate unrelated disease. We can say with confidence that, at least in the varieties studied, Para-crinkle and curly dwarf are due to the same causative agent, and that the difference of expression is simply an effect of the environment. We have as yet only incomplete evidence as to the behaviour of Crinkle "A" in Arran Victory when unmixed with other infections and grown outside, but such plots as we have examined show that its reaction under such condition is dissimilar (1928, Plot 155) to that of Para-crinkle.

The symptoms of Crinkle "A" (6) and Para-crinkle in Arran Victory may now be compared. Clearing of veins as an early symptom is common to both, mottling in Crinkle "A" begins at the junction of main veins and extends along them (6, fig. 4); it is usually not very extensive or bright, and tends to fade rapidly. In Para-crinkle the mottling also begins at the junction of veins but rapidly develops into a more or less circular, blotchy, and bright marking. Deformity may be absent in Crinkle "A" and is never very pronounced; in Para-crinkle it is an important feature and is often so extreme as to render the leaf unrecognisable. Waving in Crinkle "A" is present but slight; in Para-crinkle it is a very characteristic feature, and involves the tip of the leaflet, so that the latter is often twisted on itself and bent downwards. Necroses, present in both diseases, are more common in Para-crinkle, especially on the under surface of the main veins and the petiole.

The late phases of the symptoms are most distinct and characteristic of each disease: in Para-crinkle the mottling fades, the waving subsides, and the rugosity becomes toned down to a slight wave-like modulation of the surface, whilst the whole leaf becomes glazed (6, fig. 5). In Para-crinkle no sort of recovery sets in; on the contrary, the symptoms tend to become exacerbated, and it is only when maturation sets in that the blotching fades away into the dull grey-green of the remainder of the leaf surface, and the extreme waving becomes somewhat straightened out by reason of the harsh dry texture assumed in the latter part of the season. From the clinical point of view, Para-crinkle in Arran Victory is a far more serious complaint than is Crinkle "A."

The reaction of Para-crinkle induced by Grafting in different varieties of Potato.

The varieties which have been examined in their relation to the virus fall into one or other of the following four classes:—

1. Those varieties which react by producing the clinical picture of a severe crinkle:

   Arran Victory, and Arran Chief.

2. Those varieties which readily produce a Mosaic instead of a Crinkle:

   Arran Comrade, Great Scot, Majestic.

3. Those varieties which do not visibly react at all, but carry the virus:

   Abundance, President, and probably Champion.

4. Those varieties which neither react to the virus nor appear to act as carriers:

   Arran Crest, Di Vernon, Epicure, Sharpe's Express.

We will examine the evidence in each of the classes:—

Group 1.—It is not necessary to discuss the case of Arran Victory any further. With regard to Arran Chief (Table VII) the infection was made successfully 5 times. Whilst the symptoms are alike in type to those in Arran Victory, they are not so severe. Notwithstanding the difference of intensity of symptoms when planted out in the following season, infected Arran Chief plants develop into perfect curly dwarfs (fig. 10). The early stage of the disease, viz., formation of the blotchy mottling and early deformity characteristic of Para-Crinkle, is well seen in fig. 8. The incubation period would appear to be very variable, but as the grafts made very early in the year, *i.e.*, in January and February, are those which have the longest period, it is possible that the incubation period in the growing season is not more than about 30 days.

Table VII.—The Behaviour of

Group.	Year.	Source of infection.	Pot No.	Name of variety examined.	Pot No.
I	1927	King Edward	20	Arran Chief	8
	1927	King Edward	26	Arran Chief	13
	1928	King Edward	1730	Arran Chief	2166
	1928	King Edward	1528	Arran Chief	413
	1929	Arran Victory	$9A_2$	Arran Chief	$9A_2$ A. Ch.
	1929	King Edward	314	Arran Chief	3
II	1928	King Edward	1528	Arran Comrade	2414
	1929	King Edward	314	Arran Comrade	4
	1929	Arran Victory (3rd year)	734	Arran Comrade	734 A. Cde.
II	1928	King Edward	1730	Great Scot	T. 2109
	1929	King Edward	314	Great Scot	6
	1929	King Edward	314	Great Scot	7
	1929	King Edward	314	Great Scot	314 Gt.S.
	1929	Arran Victory (3rd year)	734	Great Scot	734 Gt.S.
	1929	Arran Victory (3rd year)	734	Great Scot	734 Gt.$S._2$
	1929	Arran Victory (seasonal)	433A	Great Scot	433 A Gt.S.
		Arran Victory (seasonal)	435A	Great Scot	435 A. Gt.$S._1$
		Arran Victory (seasonal)	435A	Great Scot	435A Gt.$S._2$
II	1929	King Edward	424	Majestic	424 Maj.
III	1928	King Edward	1730	Abundance	2157
	1928	King Edward	1528	Abundance	2415
	1929	King Edward	314	Abundance	314 Ab.
	1929	Arran Victory (3rd year)	734	Abundance	734 Ab.
III	1929	Arran Victory (3rd year)	734	Champion	734 Champ. 1
III	1928	Arran Chief	2166	King Edward	1371/3
	1928	Arran Victory	2249	King Edward	1371/2
	1929	Arran Victory (3rd year)	734	King Edward	734 K.E.
IV	1929	King Edward	314	Sharpe's Express	2
	1929	King Edward	314	Sharpe's Express	154
	1929	Arran Victory	221	Sharpe's Express	158
	1929	Arran Victory (3rd year)	734	Sharpe's Express	734 S.E.
IV	1928	King Edward	1528	Epicure	2411
	1929	King Edward	1528	Epicure	1
	1929	King Edward	314	Epicure	174
	1929	Arran Victory	221	Epicure	312
	1929	Arran Victory	734	Epicure	734 E_1
IV	1929	Arran Victory	221	Arran Crest	221 Ct.
IV	1929	Arran Victory	734	Di Vernon	734 Di

* The King Edward tested was in fact a sister plant of 1371,

various varieties to Para-crinkle.

Symptoms induced in variety examined.	Incubation period.	Field plot of variety examined.	Regraft to Arran Victory.		Regraft to President.	
			In pot.	In field.	In pot.	In field.
A rather mild crinkle	50	—	—	—	—	—
Crinkle	34	—	Scion† mosaic	—	—	—
Crinkle	50	Curly dwarf	Para-crinkle	Curly dwarf	Healthy	Healthy
A rather mild crinkle	28	—	—	—	—	—
A rather mild crinkle	29	—	—	—	—	—
Scion feeble—none	—	—	Healthy	—	—	—
Mild mosaic	28	—	—	—	—	—
Mild mosaic	37	—	Para-crinkle	—	—	—
Mosaic	37	—	—	—	—	—
None	—	Mosaic	—	—	—	—
Mosaic	34	—	Para-crinkle	—	—	—
Mosaic	49	—	Para-crinkle	—	—	—
None	—	—	None	—	—	—
Mosaic	38	—	—	—	—	—
None	—	—	—	—	—	—
Severe mosaic	24	—	—	—	—	—
Severe mosaic	30	—	—	—	—	—
Scion poor—suspicious	—	—	—	—	—	—
Severe mosaic	? 31	—	—	—	—	—
None	—	Healthy	2125 Para-crinkle 2196 Para-crinkle	—	Healthy	—
None	—	—	—	—	—	—
None	—	—	Para-crinkle	—	—	—
None	—	—	—	—	—	—
None	—	—	—	—	—	—
None	—	Healthy	Para-crinkle*	—	—	—
None	—	Healthy				
None	—	—	—	—	—	—
None	—	—	—	—	—	—
None	—	—	Healthy	—	—	—
None	—	—	Healthy	—	—	—
None	—	—	—	—	—	—
Suspicious	—	—	—	—	—	—
None	—	—	—	—	—	—
None	—	—	—	—	—	—
None	—	—	Healthy	—	—	—
None	—	—	—	—	—	—
None	—	—	—	—	—	—
None	—	—	—	—	—	—

viz., 1368, which in 1929, as 425, reacted to Arran Victory.

Group 2. *Arran Comrade.*—This variety (Table VII) was infected 3 times, the effect being a mild mosaic. The incubation period was 30 days. Re-grafted to Arran Victory the full symptoms of Para-crinkle were developed.

Great Scot.—The reaction of this variety to Para-crinkle is somewhat variable ; a severe mosaic, a mild mosaic, or even no reaction, may result from infection by grafting. The incubation period is 35 days. Nine grafts were made.

The mosaic produced in Great Scot is shown in fig. 11 : it starts like a crinkle by a clearing of the veins and an extension at their bifurcations ; the subsequent development of the paler areas, however, is rather like an inter-veinal mosaic ; later in the season the symptoms tend to disappear. Although the disease is reduced, in its clinical aspect, in Great Scot as compared with its expression in Arran Victory, the virus is not modified, as is shown by the regrafts to Arran Victory. It is possible that Great Scot may offer some actual resistance to infection, for in the case of the experiment 314 G.S. there was no fault with the scions, yet no sign of disease appeared either in Great Scot or on the regraft to Arran Victory. Great Scot gave variable results when infected by grafting with Crinkle "A."

Majestic.—Only one plant of this variety, itself of a clone which the Institute has isolated as virus free, was grafted, and reacted with a severe mosaic to the virus of Para-crinkle, Table VII.

Group 3 contains those varieties in which the disease is entirely latent. The rôle of President and King Edward have been fully dealt with (see Tables III and VI); we have, however, evidence in respect to Abundance and Champion which would lead us to place them in this group (see Table VII).

Abundance.—In this variety Para-crinkle produces no symptoms, either in the glasshouse or in the field ; the virus, however, lies latent and unaltered within, ready on regrafting to Arran Victory to evoke the full symptoms of the disease.

Champion.—Only one plant was grafted with a Para-crinkle scion, and this was derived from our most virulent source, the third season growth, 734. No reaction followed, although the scion lived on for two months and the stock flourished exceedingly. Till Champion has been regrafted to Arran Victory there is no certainty that this variety belongs here rather than to Group 4, but the expectation is in favour of its being a true carrier.

Group 4.—This group contains, so far as we have ascertained, the varieties Sharpe's Express, Epicure, Arran Crest and Di Vernon. The last two are placed here rather than in Group 3 by analogy, for they have not been yet

successfully regrafted to Arran Victory, and must wait further investigation. It is of interest that all the members of the group are first earlies, and appear to resist infection with Para-crinkle. Two of them, viz., Sharpe's Express, and Di Vernon, behaved in a similar manner towards infection with Crinkle " A."

The infections by Para-crinkle, so far described, have been effected by means of grafting ; efforts were made to obtain similar results by inoculation of juice. The method was the same as that used in the case of Crinkle " A " (6) and consisted in pounding up a few leaves of the affected plant in a sterile mortar, scratching and rubbing the same into three leaves of any plant under trial with a sterile needle. In Table VIII will be seen the results. In six cases control grafts were made with scions cut from the inoculated plants to healthy Arran Victorys : in none was there any reaction. In all, 32 inoculations were made into 11 different varieties. Analogy with the results obtained by grafting suggest that visible results might have been expected in the case of Arran Chief, Arran Comrade, Great Scot and Arran Victory. In fact, no response to the inoculations was found in any of these varieties, with the exception to be discussed directly, or in those other varieties which grafting experiments proved to be symptomless carriers.

In regard to the results of inoculation to Arran Victory there arises a point

Table VIII.—Inoculation of Para-crinkle to various Potato varieties.

Year 1929.	Source of infection.	Pot No.	Variety inoculated.	No. of Plants.	Result.	Regraft to Arran Victory result.
1929	King Edward	314	Abundance	1	None	None
1929	King Edward	314	Arran Chief	1	None	None
1929	Arran Victory	221	Arran Comrade	1	None	
1929	Arran Victory	221	Arran Crest	1	None	
1929	Arran Victory	221	Arran Victory	3	None	
1929	Arran Victory	424A	Arran Victory	3	None	
1929*	Arran Victory	499A	Arran Victory	1	None	
1929	Arran Victory	734	Arran Victory	1	Para-crinkle	
1929	Arran Victory	734	Arran Victory	1	None	
1929	Arran Victory	734	Arran Victory	1	Severe Mosaic	
1919	King Edward	314	Champion	1	None	None
1929	King Edward	314	Epicure	1	None	None
1929	Arran Victory	221	Epicure	1	None	None
1929	King Edward	314	Great Scot	1	None	None
1929	King Edward	314	King Edward	1	None	
1929	Arran Victory, 3rd year	734	President	4	None	
1929	Arran Victory	424A	President	3	None	
1929	Arran Victory	221	President	3	None	
1929	King Edward	314	Sharpe's Express	1	None	
1929	Arran Victory	221	Sharpe's Express	1	None	

of much interest, and perhaps importance. Seven inoculations were made from sources whose own infection was recent, *i.e.*, of the same season : in all 7 no sign of any disturbance was seen on the inoculated plants ; in the next 3 infections the source was an Arran Victory Para-crinkle plant (734) whose infection dates back three years ; in this case we find 2 out of the 3 plants showing a very definite reaction ; in one a typical Para-crinkle, in the other a severe mosaic.

It will be remembered that when President was grafted from this source, 1 out of the 6 plants treated developed a mosaic which, on grafting back to Arran Victory, produced in the latter Para-crinkle. It would appear, therefore, that the virulence of Para-crinkle in a highly susceptible plant, such as Arran Victory, tends to increase when perpetuated by tuber from year to year. Needle inoculation as a method of infection is obviously more akin to that arising from the attacks of sucking insects than is that by grafting, and although it would appear to be more or less unimportant as a means of conveying the virus of Para-crinkle, yet if the virulence of the virus is commonly exalted by growing on by tuber from season to season, then it will be perceived how readily an apparently harmless " groundkeeper " might become a potent source of infection in the field.

The King Edward sources were all from infected tuber-born plants, but unlike the Arran Victory plants of like origin they failed to effect an infection. There would appear to be a difference between the carrier plant and the affected plant in this relation.

In the case of Crinkle " A " it was shown how inoculation methods including imbibition produced mild mosaic in the inoculated plants instead of crinkle. In the series of inoculations in Table VIII we seem to be on the border line between inoculations which fail to achieve any infection, and those which by reason of an increased virulence just overstep the threshold of resistance in the plant and produce a reaction. Obviously more research is needed on this point, but it would appear that on the one hand there is some, albeit a feeble, resistance to infection to the virus on the part of even susceptible plants, and that on the other, the virus itself may by continued residence within a susceptible plant acquire a greater initial power of infectivity.

The Reaction of Datura to Para-crinkle.

Crinkle " A " when communicated to Datura by inoculation produces a characteristic reaction which has been already described (6). Inoculation of Para-crinkle, however, fails to produce a reaction whether it be introduced

directly from King Edward, or indirectly from it through Arran Victory; even in the case of Arran Victory (734) where we have seen there is reason to believe the virulence of the virus has been augmented.

The sources of Para-crinkle infection used were 2 King Edwards inoculated into 10 Daturas; 1 Abundance into 5 Daturas; 2 Arran Chiefs into 6 Daturas; 8 Arran Victorys into 42 Daturas; 1 Great Scot into 5 Daturas; and 3 Presidents into 15 Daturas.

In all, 83 plants of Datura were inoculated, with negative results. This result may be compared with that obtained by inoculation of Datura with Crinkle "A," in which case, with a like number of plants, there is close on 100 per cent. of successful reactions. In the reaction of Datura following inoculation we have a valuable criterion for distinguishing between the two types of crinkle.

The uniformly negative results, together with the failure to obtain a subsequent reaction in Arran Victory or Datura by further *passage*, seems to be good evidence for the fact that Datura is resistant to the virus of Para-crinkle when introduced by the needle.

Notwithstanding the facts recorded and the observations just made, we have some evidence which shows that under certain conditions Datura may react to needle inoculation. The first case concerns the virus of Para-crinkle as derived from the three-year old Arran Victory source 734. When this was grafted to six Presidents, one of these developed a mosaic, the remainder nothing. Inoculation of the juice from this one plant to five Daturas produced in them a reaction which, weak at first, gradually approximated in strength to that seen in Crinkle "A" infection. Although there has been some reason to suspect the initial condition of this particular President plant, the result does not stand alone and cannot be disregarded. One of the five Daturas directly infected by needle from 734 developed a peculiar mottling (fig. 12, Plate 15) very similar to that seen in Daturas on grafting, which, however, faded away.

The second case is less equivocal. The source of the Para-crinkle was a freshly infected Arran Victory (221): a scion of this *grafted* to a Datura produced a reaction which we shall see is peculiar to Para-crinkle in Datura, when infected by grafting with this virus. From the Datura the disease was passed by grafting to an Arran Victory, in which it produced a peculiar mosaic, a pale diffuse mottling with white necrotic spots on the veins.* A further *passage* was made through another Arran Victory, with the production of a similar mosaic, only without necrotic spotting, and from the latter plant inoculations were

* *Cf.* p. 168. Similar reaction after passage by grafting through Datura.

made to five Daturas. All the Daturas responded and produced a mottling in their leaves (fig. 13, Plate 16) which is distinct from that caused by Crinkle "A" in that it is more or less confined to the base of the leaf, is far less vivid, and the pale areas lie immediately alongside the veins, almost completely fill in the interveinal areas, and thus prevent the formation of the green lines characteristic of Crinkle "A." Rugosity of the leaf, often a prominent symptom of Crinkle "A," is here absent or minimal.

The above facts warrant the belief that although Datura is resistant to infection by the virus of Para-crinkle as a consequence of needle inoculation from the potato, yet *passage* of the same through Datura, and again through Potatoes, increases its virulence sufficiently to assure its gaining a foothold in Datura when introduced by needle. Datura, resistant as it is to Para-Crinkle when introduced by needle infection, can however be infected by means of grafting.

Infection of Datura by means of Grafting.

There is no difficulty in grafting Datura with normal or diseased Potatoes, and in the case of Para-crinkle this has been done seven times (Table IX). Symptoms appear in 13–14 days, and at every stage they differ from those that were recorded for Crinkle "A" (6). In Crinkle "A," clearing of veins is an early and pronounced symptom: here it is hardly noticeable; mottling in Crinkle "A" begins at the base of the leaf and starts as a fine network of cleared veins; here it generally begins at the apex and is a soft, diffuse, rather large type of mottle not associated with the veins (fig. 14). This stage lasts a little time, the mottling becomes more diffuse, and fills the main interveinal areas; but as the actual loss of colour is often but little, it is not always easy to differentiate the morbid leaf from the normal. Later there supervenes a new and distinct character not seen in infections with Crinkle "A." This new feature is the development of numerous small bright yellow spots which are more or less densely clustered towards the apex of the leaf (fig. 15). The spots persist for about a month and then fade away, usually leaving the leaf with a faint diffuse mottling. New leaves arising at this period do not develop the spots, but present once more a mottling, which is less patchy than the primary one and more distinctly interveinal (fig. 16).

The symptoms following grafting with the old stock of Para-crinkle 734 are the same as those from freshly inoculated sources; but those from 734, after passage through the one President plant which reacted to this source on needle inoculation, are rather more distinctive. The clearing of veins is more pronounced, the early mottling brighter and larger and accompanied by an almost

imperceptible fine network of faint white lines at the apex of the leaf (fig. 12). The spotting phase follows, but the spots are for the most part small ring-shaped structures (fig. 17); these in their turn fade, and the final stage is, at least in many leaves of the plant, a much more bold mottling, which is largely interveinal (fig. 18), but where it approaches closely to the veins it does not leave that narrow dark green line which is seen in the case of Crinkle "A." The clinical picture in this case resembles that seen in fig. 13, where intensification of the virus was secured by consecutive passage through Datura and Potato.

It will be observed in Table IX that in one case, viz., Pot 233, the source was a President scion, which was carrying but not exhibiting any symptoms whatever of the disease, but which nevertheless produced a reaction in the Datura which however did not include the formation of spots. The scion in this case died at the end of three weeks, which may account for this omission. President carrying Para-crinkle from the 734 source did produce when grafted to Datura the entire gamut of symptoms.

Table IX.

Year.	Source.	Pot No.	Effect on Datura of Potato graft.	Incubation period. Days.	Datura Pot No.	Result of regrafting to Arran Victory.	Pot No.
1929	President (carrying Para-crinkle)	233	Faint interveinal mottle from which it recovered	10	777·3		
1929	Arran Victory	$9A_2$	Faint mottle which persisted	20	777·2		
1929	Arran Victory	221	Interveinal mottling and subsequent yellow spotting	19	221 Dat. 1		
1929	Arran Victory	221	,, ,,	15	221 Dat. 2	Mosaic with fine necroses	221 Dat. 2A.
1929	Arran Victory, 3rd year	734	,, ,,	15	849·1		
1929	Arran Victory, 3rd year	734	,, ,,	15	850·3	Mild Mosaic in all 3 plants	$850{\cdot}3A_1$, $850{\cdot}3A_2$, $850{\cdot}3A_3$
1929	President (carrying 3rd year Arran Victory 734)	734P	,, ,,	17	734P Dat.		

The passage by grafting through Datura of the virus of Para-crinkle appears to reduce its virulence, for in the four cases where the infected Datura was regrafted to Arran Victory the result was not Para-crinkle but a mild mosaic,

with, in one case, the formation of a few minute white necrotic spots. A similar reduction of virulence was observed when Crinkle "A" was passed through Datura, which in this case can be readily effected by needle inoculation as well as by grafting.

The problem of what is the nature of the action of the Datura on the Potato virus was discussed in regard to Crinkle "A." The problem there was complicated by the fact that there was some evidence for considering Crinkle "A" as a mixture of viruses, and the possibility of Datura being a selective as well as a modifying agent arose. In the case of Para-crinkle the problem appears simpler; its reaction, so far as we have explored it throughout the varieties of Potato, is all of one kind—a crinkle-mosaic type. Datura, when it reacts on it, reduces the crinkle to a mosaic; whether permanently or no, remains to be seen; and when it fails to react on it, it is because it totally excludes the virus.

How does the Datura act in the presence of the Para-crinkle virus? Is the failure to become infected on needling due to a reaction in the leaf, a neutralization of the virus locally, or is the reduction of virulence after *passage* through Datura by grafting due to the action of some protective substance manufactured in the leaves, and if so, is it a normal constituent of Datura tissues? To attempt to answer this question the senior author carried out a series of "double" grafts similar to those employed in the case of Crinkle "A."

The method is to graft to a healthy Arran Victory a piece of Datura stem 2 inches long and from ¼ inch to ½ inch in diameter. In one series the Datura stem is cut from between the nodes, and in the other it includes a node and its leaves within its length. At the same time or later a Para-crinkle-infected scion of Potato is grafted to the Datura scion's distal end. In order to secure success very special care is needed to preserve the plants in a moist warm atmosphere for about ten days, after which they do well.

With one exception, which will be dealt with directly, the presence of leaves on the Datura stem *prevents* any virus reaching the plant—the leaf neutralises or in some way destroys it—for we know that the virus must have entered the living Datura stem at its junction. Absence of leaves on the Datura stem, and the passage of the Para-crinkle virus through a solid dense mass of Datura tissue, have no effect whatever on the virus, but allow of the full and ready infection of the basal Arran Victory stock (see fig. 3).

The form of the double graft in which there are no Datura leaves is shown in fig. 19, Plate 17. The results are shown in Table X. Four double grafts were made in which Datura was devoid of leaves. All of these developed Para-crinkle.

Table X.—" Double Grafts " infections of Arran Victory through Datura.

Year.	Source of infection.	Pot No. of source.	Date of graft of Datura to stock.	Date of graft of infected Potato to Datura.	Datura stems with leaves.	Datura stems without leaves.	Incubation period. Days.	Pot No.	Effect on Arran Victory stock.	Condition of Scions. Datura.	Condition of Scions. Potato.
1929	Arran Victory	5A	22/3	2/5		+	16	217 A_1 ...	Para-crinkle ...	Healthy	Poorly
1929	Arran Victory	793	10/7	10/7		+	19	793 Dat. A ...	Para-crinkle ...	Healthy	Grown
1929	Arran Victory (3rd year)	734	10/7	10/7		+	46	734 Dat. A ...	Early signs of Para-crinkle recovered	Healthy	Grown
1929	President (carrying Para-crinkle)	424P	11/7	11/7		+	33	424P Dat. A...	Para-crinkle ...	Healthy	Very strong
1929	Arran Victory	5A	5/4	23/5	+			217 A_3 ...	None	Healthy	Grown
1929	Arran Victory	229	5/4	23/5	+			229 Dat. A_2 ...	None	Healthy	Poorly
1929	Arran Victory	424A	15/7	15/8	+			424A Dat. A	None	Healthy	Poorly
1929	President (carrying Para-crinkle)	424P	16/7	27/8	+			424P Dat. A_3	None	Healthy	Grown
1929	Arran Victory (3rd year)	734	15/7	15/8	+		32	734 Dat. A_2 ...	Suspicious ...	Mottled	Grown
1929	Arran Victory	424A	15/7	15/8	+		46	424 Dat. A ...	Para-crinkle ...	Healthy	Grown

One plant, 734 Dat. A, developed symptoms very late and over only a small area, which subsequently recovered almost entirely; the remainder showed Para-crinkle at its fullest. Fig. 3 shows the stock plant of the double graft 217 A_1 in an early stage of its seizure with Para-crinkle.

The infection of the Arran Victory stock of the virus after passage through 2 inches of solid stem is at least evidence that the normal tissues which make up the Datura stem contain no antibody or other neutralising agent in respect to Para-crinkle. It will be noted that the carrier President scion is equally effective as an infective agent under these circumstances as any displaying the active symptoms of the disease.

In the second series, where the Datura has leaves, there are six double grafts. In four there is no reaction whatever in the stock plant. In one, 734 Dat. A_2, nearly two months after the second graft, a few blotchy crinkle-like markings appeared too late in the season to allow of further development. Now this case, like the next, is the exception which goes to prove the rule that the presence of Datura leaves on the graft prevents the virus of Para-crinkle from becoming effective. In this plant the Datura leaves matured early, leaving the Potato scion still active—a thing which did not occur in the other "double" grafts, either in the Para-crinkle or the Crinkle "A" series, and it is probably to this maturation of the Datura leaves that should be ascribed the free passage late in the season of the virus down the stem and past the node into the Arran Victory stock.

Plausible as this explanation may be, it should however be noted, that the scion which produces this exceptional result is from pot plant No. 734, the old Arran Victory, whose reactions have always shown a certain individuality, which is best interpreted as evidence that the virus of Para-crinkle increases in virulence from year to year when grown on by tuber. The second exception is the "double" graft 424 Dat. A.; in this case the reason for the failure of the action is clearer. In the other grafts the Datura stem was a perfectly straight rod-like section (fig. 19); in this, there is (fig. 20) a forked branch on an almost bifid stem. On one branch of the fork is the Potato scion, on the other the Datura leaves. The enlargement of the forked stem shows that it is highly probable that the conducting vessels from these two branches never met at all before the Potato stem itself was reached, and that therefore the virus could attack the tissues of the Potato without any mixture *en route* with the products of metabolism from the Datura leaves.

As in the case of Crinkle "A," so here. It is the natural metabolic activity of the growing Datura leaf which furnishes the body, whatever it may be, that

reacts on the virus; in the case of Crinkle " A " it brings about attenuation; in that of Para-crinkle it either neutralises or destroys it.

The Reaction of Tobacco to Para-crinkle.

The juice of infected Para-crinkle plants have been inoculated by needle many times to Tobacco. In no case has there been any reaction—a result which adds a further distinction between this virus and that of Crinkle " A." Two varieties of Tobacco, Virginian and White Burley, have been employed. The latter so sensitive to common Potato mosaic and Crinkle " A " is as unresponsive as the former.

The details of these inoculations are as follows: Five Arran Victorys were inoculated into 22 White Burleys; 3 Arran Victorys into 32 Virginians; 1 Arran Chief into 1 Virginian; 1 President into 12 Virginians; and 2 Daturas inoculated with Para-crinkle into 10 White Burleys.

Grafts of infected Arran Victory scions were made to both varieties with negative results.

The investigation of Para-crinkle, of which this account is a record, is incomplete in several important particulars. Time has not yet allowed of adequate research into the possible environmental factors in the field, which may either modify its expression in varieties which react under glass, or evoke symptoms in those varieties which are carriers under like conditions. It is true that we have been able to show that Para-crinkle in Arran Victory and Arran Chief assumes the appearance of curly dwarf when growing outside, but what happens in other varieties—if anything—is yet to be determined. Sometimes President, when carrying the virus, has looked " suspicious," and it would not be surprising if under consistently adverse conditions it and other infected carrying varieties might not exhibit a mosaic or other lesion.

So far, no success has attended the initial efforts of my colleague, Dr. Kenneth Smith, to convey the disease by aphids. Research on this line it is hoped may be pushed forward in the coming season.

This paper, though dealing with what has been shown to be a distinct pathological entity, should be read in conjunction with the senior author's paper on Crinkle " A." From the consideration of both papers, it will be apparent that the clinical disease of Crinkle can no longer be regarded as a unit disease. It may be due to either the virus of Crinkle " A " or that of Para-crinkle, and what is perhaps of equal importance, is that many of the simple mosaics we meet with in the field are not " simple mosaics " at all, but forms of either of the

two Crinkles as modified either directly by the variety infected, or indirectly by the plant from which the infection was transferred.

We wish to thank Miss O'Connor for her care of the pot plants, and Mr. Cory for much assistance during the progress of the work.

Summary.

The presence of virus diseases in Potato plants of the King Edward variety which appear to be in perfect health, is demonstrated.

One of the latent diseases in King Edward is due to a pathogen which produces in the variety Arran Victory a violent crinkle. This disease, the subject-matter of this paper, has been named Para-crinkle.

A discussion of the virus-disease problem in Potatoes follows, in which such points as the possibility of acquiring stocks of the varieties to be examined, and for research purposes the absolute necessity of maintaining them virus-free are stressed.

No support is found for the existence of viruses in really healthy Potatoes.

The variable reaction of different varieties to the same pathogen is described and the practical importance of the absence of reaction to infection is shown.

The problem of the effect of environment on the tuber-seed of the Potato is touched on, and the opinion put forward that this effect is in reality due to the reaction of the environment on a latent or partially suppressed virus disease, and that completely healthy virus-free potatoes may prove physiologically stable within very wide fluctuations of environmental conditions.

The Para-crinkle latent in the two varieties King Edward and President is studied, and the occasional differences between their behaviour in the glasshouse and in the field pointed out.

Arran Victory plants are as readily and as severely diseased by infecting from a " carrier " plant as from a visibly diseased plant of its own variety.

The symptoms of Para-crinkle in Arran Victory are described, both in the seasonal infection and in the tuber-born plant, and compared with those of Crinkle " A."

The reaction of several other varieties to Para-crinkle is described. They fall into four classes: those which react like Arran Victory with a crinkle; those which react with a mosaic; those which carry the virus but do not react visibly to it; and those which neither react to nor carry the virus. In all these experiments infection was attempted by means of grafts.

Eleven varieties were inoculated by needle with the juice of Para-crinkle

infected plants with no result, whether the infective source was derived from a plant infected recently, or from King Edward where it was from infected tuber-born plants. In the case where Arran Victory was the source and itself several seasons old, an infection was effected.

Inoculation of Para-crinkle by needle to Datura is without effect, nor is there any evidence that it exists in a latent condition in the Datura. The virus, however, succeeds in entering under certain conditions. Thus, when the virus is derived from a tuber-born diseased Arran Victory plant a reaction does occur; again, when the virus has been passed by grafting through Datura and then through Potatoes, and again by needle to Datura, a reaction was obtained. The reactions thus obtained are distinct from those following needle inoculations with Crinkle "A," as well as from those obtained by grafting Para-crinkle-infected scions.

Datura can be infected with Para-crinkle by grafting scions of infected and visibly diseased plants, and equally by grafting scions of "carriers." The symptoms induced are characteristic and distinct.

Double grafts made by grafting Datura, with and without leaves attached, to Arran Victory and a Para-crinkle scion to the Datura, demonstrate that passage through the tissue of a solid Datura stem, 2 inches long, has no effect on the virus, but that the presence of the leaves and the mixture of their metabolic products in the stem tissue with the virus passing through it destroys the virus.

Neither inoculation of juice nor grafting of Para-crinkle scions has any effect on Tobacco plants of the two varieties tried.

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EXPLANATION OF PLATES.

Plate 13.

Fig. 1.—King Edward plant: healthy to all appearance, but carrying Para-crinkle.

Fig. 2.—An Arran Victory plant grafted with a President scion carrying Para-crinkle. The scion, which has grown vigorously, is perfectly healthy in appearance; the Arran Victory stock is showing severe crinkle.

Fig. 3.—The top of an Arran Victory stock in which crinkle symptoms have developed very rapidly as a result of a "double" graft through a Datura leafless stem.

Plate 14.

Fig. 4.—Leaf from a Para-crinkle infected Arran Victory plant.

Fig. 5.—Leaf from a Para-crinkle infected Arran Victory plant. Showing deformity.

Fig. 6.—The top of a Para-crinkle infected Arran Victory plant, showing advanced symptoms.

Plate 15.

Fig. 7.—A plant of Arran Victory raised from tuber which has been infected two years previously with Para-crinkle.

Fig. 8.—Leaf from a Para-crinkle infected plant of Arran Chief, showing the early symptoms of the disease.

Fig. 9.—An Arran Victory plant grown in the field raised from the tuber of a plant infected in the previous season: the plant has developed "curly dwarf."

Fig. 10.—An Arran Chief plant grown and infected under like conditions to that in fig. 9.

Fig. 11.—A leaf of Great Scot, showing mosaic following infection by Para-crinkle.

Fig. 12.—Datura leaf, following on inoculation from the three-year-old infection of Para-crinkle in Arran Victory.

Plate 16.

Fig. 13.—Datura leaf: showing the mottling arising from inoculation of Para-crinkle after repeated *passage*.

Fig. 14.—Datura leaf: showing the early symptoms of Para-crinkle infection following grafting.

Fig. 15.—Datura leaf: showing the spotting which follows on the mottling seen in fig. 14.

Fig. 16.—Datura leaf: the mottling seen on the same plant on leaves developing after infection.

Fig. 17.—Datura leaf: showing an accentuation of the symptoms shown in fig. 15 following inoculation of Para-crinkle after passage through President from the three-year-old infected stock of Arran Victory.

Fig. 18.—Datura leaf: showing symptoms which follow on those seen in fig. 16.

FIG. 1.

FIG. 2.

FIG. 3.

(*Facing p.* 174.)

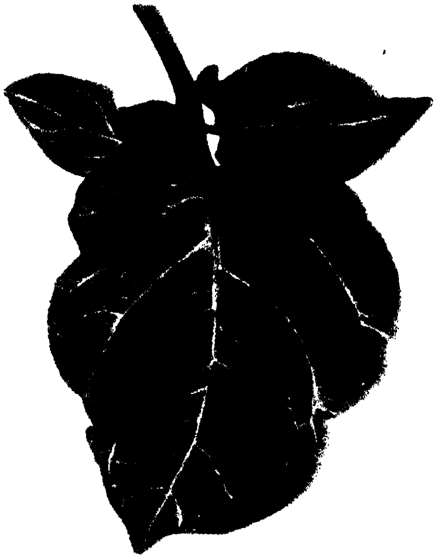


Fig. 5.

Fig. 4.

Fig. 6.

FIG. 7.

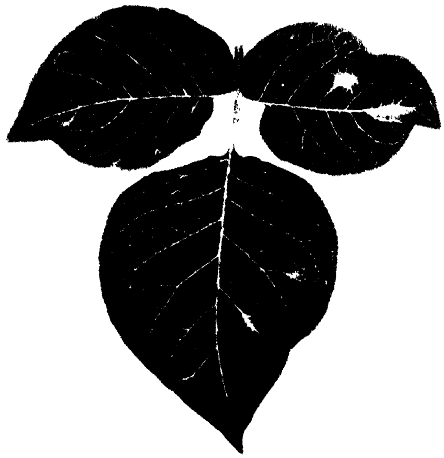
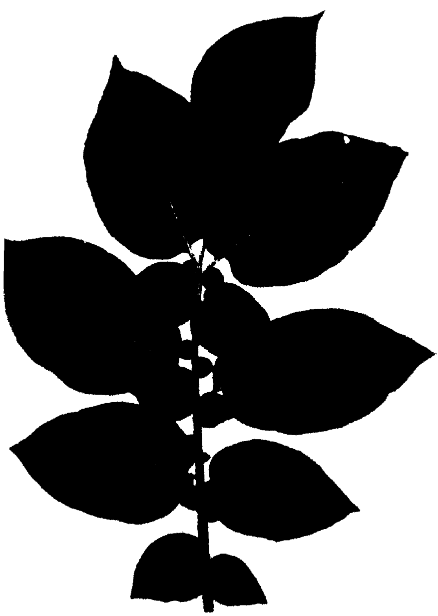


FIG. 8.

FIG. 9.

FIG. 10.



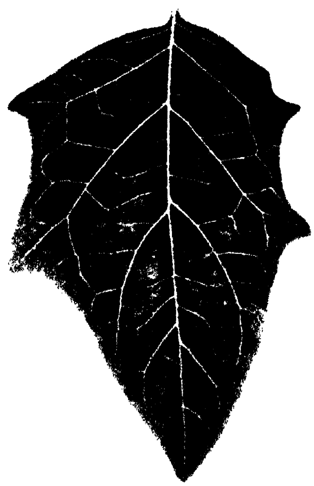


FIG. 12.

Fig. 13.

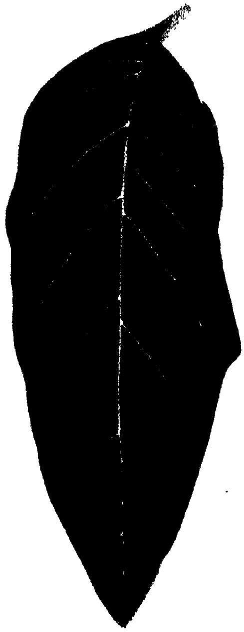


Fig. 14.

Fig. 15.

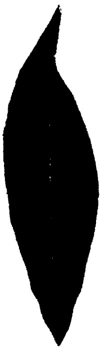


Fig. 16.

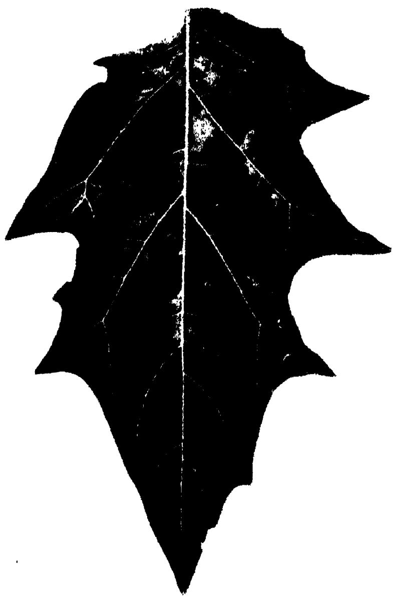


FIG. 19.

FIG. 20.

FIG. 21.

PLATE 17.

FIG. 19.—A "double" graft without leaves, *i.e.*, infected Para-crinkle Arran Victory scion on Datura stem on to Arran Victory stock; the leaves of the stock plant are beginning to show symptoms of Para-crinkle.

FIG. 20.—A "double" graft with leaves borne on a forked stem. Para-crinkle has gone over to the Arran Victory stock.

FIG. 21.—Enlargement of the forked Datura stem showing that the two branches are still visibly distinct in the main stem.

612.014.467

Observations on the Movement of Cells in vitro.*

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(Communicated by Prof. J. T. Wilson, F.R.S.—Received January 9, 1930.)

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[PLATE 18.]

Introduction.

The term chemotaxis has been applied to a phenomenon in which the movement of certain living cells may be controlled by a concentration gradient of certain definite chemical substances in a fluid medium. It was shown by Pfeiffer (1) that a directive movement of the motile gametes of ferns could be ascribed to the action of a substance in solution and similar movement could be obtained by means of malic acid. Dakin and Fordham (2) following a similar technique showed that echinoderm spermatozoa migrate into capillary tubes containing an extract of unfertilised eggs to a greater extent than into control tubes, and the phenomenon cannot be ascribed solely to capture. The present paper is an extension of some preliminary observations (3) in which the cells migrating from a fragment of chick spleen grown *in vitro* in amniotic fluid were found to move to or from a piece of marble containing a trace of absorbed substance.

* This work was carried out under the control of the Cancer Research Committee of the University of Sydney, and with the aid of the Cancer Research and Treatment Fund.

Technical Procedure.

Following the previous technique a fragment of spleen about ½ mm. square was obtained from a 14-day chick embryo and placed on a coverslip in a fairly large drop of amniotic fluid from the same embryo. In the present experiments porous tile was substituted for marble (3) and fragments of approximately the above dimensions were soaked for some days under aseptic conditions in saturated solutions of the following: glucose, sodium chloride, potassium chloride, calcium chloride, uranium nitrate and also in embryo extract and normal saline as a control.

The prepared fragments were washed for 20 minutes in saline and also for a few minutes in a drop of the growth medium so as to remove any large amount of " foreign substance " adhering to the surface. In some cases referred to as "strong preparation," only a very brief washing was given. The prepared fragments were then placed individually on the coverslip droplets about 3 mm. away from the spleen fragment and the drop was drawn off until very thin, so that both the fragments were held firmly in place by the force of surface tension. The preparation was then sealed in a cavity slide and after a preliminary observation was incubated for 24 hours without any disturbing movement.

Experimental Results.

The cultures were usually set up six at a time, at least two of which were control preparations. The bulk of the work was carried out with glucose preparations and the results are recorded in the following table. The term neutral implies a perfectly symmetrical migration of cells from the spleen after incubation (Plate 18, fig. 1). The term " positive " implies that a definite unidirectional movement of cells was observed towards the tile fragment, giving in favourable cases a continuous column of cells in the intervening space (fig. 2). There are several fallacies in the interpretation of such an appearance and it is particularly necessary to observe and roughly sketch the preparation as soon as it is set up, as a mass of cells may be spread out accidentally. A spurious result may also be obtained if the spleen and tile fragments are placed close together. Surface tension then determines a " ridge " of fluid connecting them and floating cells may flow into this. True migration may be confined to the cells which remain adherent to the coverslip and as far as possible all fallacies have been excluded from the recorded results.

The term negative implies a migration away from the tile fragment, which was observed when a large amount of dissolved substance was introduced into

Fig. 1.—Control.

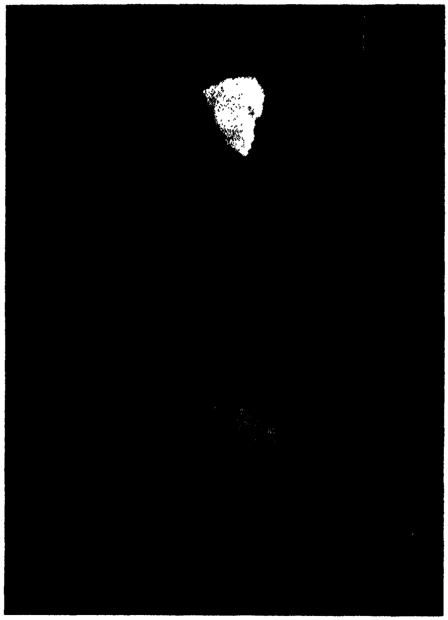

Fig. 2.—Positive Movement.

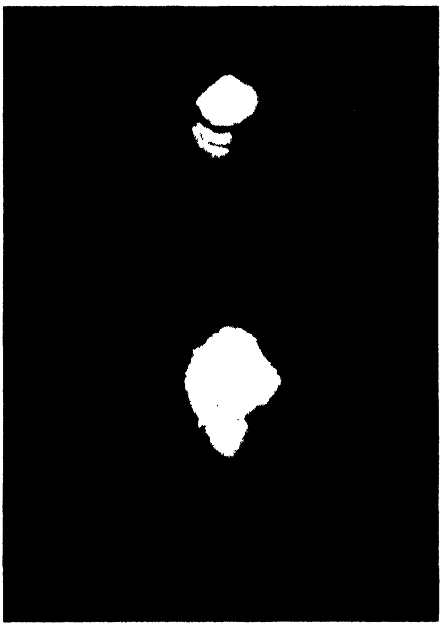

Fig. 3.—Negative Movement.

(*Facing p.* 176.)

the culture (fig. 3). The conditions were varied somewhat; if the tile was washed for 1 or 2 minutes a positive movement was obtained, whilst a more brief treatment gave a condition of balance, with no movement, and negative movement was only observed when the tile was heavily charged with foreign substance. The consequent alteration of the growth medium leads to the presence of a large number of dead cells and products of disintegration, so that many such specimens could only be classified as unsatisfactory.

With the above limitation negative movement of a fairly definite nature was observed in eight specimens, and also in three specimens employing sodium chloride. A positive movement is recorded in the table with weak preparations of sodium chloride and calcium chloride, potassium chloride, uranium nitrate, glycerin and embryo extract and quinine hydrochloride was mentioned in the earlier paper (3).

	Total.	Neutral.	Positive.	Negative.	Unsatis-factory.	Reliability.
Control	30	27	3	—	—	27/30
Glucose, weak	33	—	31	—	2	31/33
Glucose, strong	26	6	2	8	10	8/26
Sodium chloride, weak	6	—	6	—	—	6/6
Sodium chloride, strong	3	—	—	3	—	3/3
Calcium chloride, weak	3	—	3	—	—	3/3
Potassium chloride, weak	3	—	3	—	—	3/3
Uranium nitrate, weak	3	—	3	—	—	3/3
Glycerine, weak	3	—	3	—	—	3/3
Embryo extract	3	—	3	—	—	3/3

Discussion.

The above results, involving over 100 experiments, amply confirm the observations made in the preliminary paper (3). In the case of a control specimen the growth is not affected by the presence of the tile fragment, except in a few instances where one may assume that some diffusible impurity remained. Migration takes place in a symmetrical fashion, as if there were some centrifugal force. Perhaps this may be determined by the diffusion of substances from the parent fragment.

The present investigation involves the addition of a unidirectional directive force, which is presumably due to a concentration gradient which is established as the diffusible substance slowly leaves the tile fragment. The actual concentration attained must be very small. Analyses of 200 fragments in the case

of a weak preparation of sodium and potassium chloride gave on an average 0·0003 mg. and 0·0005 mg. per fragment respectively.

Taking the droplets as 1 cubic millimetre the maximum concentration attainable would be of the order of 0·05 per cent. apart from chlorides naturally present in the growth medium. From the similar technical procedure it may be assumed that all the substances tested attain a concentration of the same order and all cause a positive movement, which was summed up by the term "curiosity" (3). The migrating cells also appear to move from a substance when the concentration becomes sufficient to be injurious.

Experiments with a known Concentration.

In order to obtain an approximate measurement of the concentration gradient associated with positive or negative movement an apparatus was made consisting of two reservoirs holding solutions of different strengths with a narrow intervening space through which diffusion occurred.

The arrangement is shown in fig. 4 and consists of a brass plate about

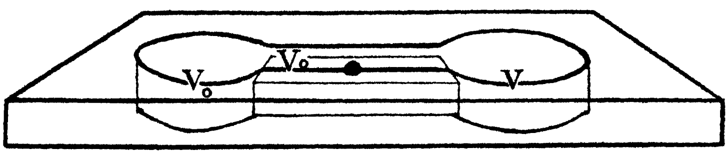

Fig. 4.

$3 \times 1 \times \frac{1}{8}$ inch, with two circular apertures, $\frac{3}{4}$ inch diameter, and a connecting channel, 1 inch long and $\frac{1}{4}$ inch wide. The whole was covered with sterile paraffin and sealed to a glass slide to close the apertures, and the height of the connecting channel was reduced to 1 mm. by means of an accessory plate. The various cavities were sealed with coverslips, and an investigation was made with coloured solutions in order to perfect a technique which would not result in a preliminary mixing of the contents.

A series of solutions were made containing 0·01 per cent. KCl, 0·02 per cent. $CaCl_2$ and NaCl, in various concentrations from 0·6 per cent. to 5·8 per cent. These values are given in Tables II and III, together with the results of a check estimation of chlorides after sterilisation.

All the experiments were made in batches of 5, of which 2 were controls in which the same solution was placed in all parts of the apparatus. In the other 3 specimens the connecting passage and one reservoir were filled with the weaker solution and the other reservoir with the stronger. Spleen fragments prepared as above were placed in the centre of the connecting passage and

drawings were made at the time of setting up, 2 hours later when one assumed the concentration gradient to be well-established and 24 hours later as a final check, the preparation being left at incubator temperature.

Table II.

	Strength of NaCl by calculation (per cent.).	Total chlorides by analysis (gm. per litre).	No.	Neutral.	Positive.	Negative.	Unsatisfactory.	Reliability.
A	(0·8)(0·8)	(0·50)(0·50)	24	22	—	—	2	41/44
	(0·9)(0·9)	(0·56)(0·56)	6	5	—	—	1	
	(1·8)(1·8)	(1·10)(1·10)	14	14	—	—	—	
B	(0·8)(0·9)	(0·50)(0·56)	15	1	14	—	—	27/30
	(0·8)(1·0)	(0·50)(0·625)	15	2	11	—	2	
C	(0·8)(1·8)	(0·50)(1·10)	9	(7 all early positive, later negative)			2	12/15
	(0·9)(2·8)	(0·56)(1·40)	6	(5 all early positive, later negative)			1	
D	(1·8)(1·9)	(1·10)(1·19)	15	—	—	15	—	21/21
	(1·8)(2·0)	(1·10)(1·23)	6	—	—	6	—	

The results are given in Table II where positive indicates movement to the higher concentration, using the above-mentioned descriptive terms. The paired brackets contain the concentration values in the two reservoirs and the controls are grouped together in the first three lines (A). Out of 44 specimens all showed a symmetrical growth except 3, which were rejected owing to the unsatisfactory growth possibly associated with infection.

The next series were associated with a nearly isotonic medium and a very low concentration gradient and the general result was a positive movement (B). When the concentration gradient was considerable, it was observed by frequent inspections that a decided positive movement was followed by a negative movement, which was presumably associated with a rising concentration (C). In the last series of Table II, if the concentration is high there is negative movement even though the concentration gradient may be comparatively low (D).

A typical specimen from each of the above groups is shown in fig. 5, for each period of observation. The preliminary sketch is just as necessary as in the tile preparations, since any clumsy manœuvre may spread a trail of cells. The average rate of migration was 1 mm. per hour, but some cells appear to move much more rapidly than others. Occasionally streams of débris were

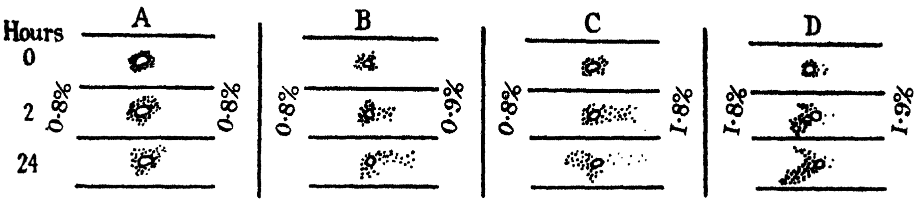

FIG. 5.

observed to spread from the fragment, often in a different direction to that of the healthy cells.

Mathematical Treatment.

A preliminary analysis was made in order to find the rate of diffusion in the vessel described. As the experimental error was considerable with the small quantities available, 0·6 per cent. and 5·8 per cent. solutions were used, samples were drawn off for analysis from each reservoir after 1, 2 and 3 hours in the incubator.

Table III.

	Time.			
	0 hour.	1 hour.	2 hours.	3 hours.
	"chlorides" mg. per c.c.	"chlorides" mg. per c.c.	"chlorides" mg. per c.c.	"chlorides" mg. per c.c.
Weak	4·0	3·3	2·8	2·8
Strong	0·4	1·2	1·7	1·6
Total	4·4	4·5	4·5	4·4

It may be assumed that in the case of the smaller differences recorded in Table II the concentration gradient is well-established after a period of 2 hours, but the concentration in the two reservoirs is not greatly altered.

In the present work one cannot determine exactly the point at which a cell begins to show a selective movement. One aims rather at a very approximate solution, which permits the full use of simplifying methods. Referring to fig. 4 one may then assume that one reservoir (V) remains constant during the experiment, while the other passage and reservoir (V_0) represent a cylindrical diffusion vessel of infinite length.

The diffusion of substances in solution takes place according to Fick's law $dV/dT = Kd^2V/dx^2$, where V is the concentration at time T in a position defined by a co-ordinate x and K is the constant of diffusion.

The following solution is adapted to the above conditions :—

$$V_x = V - (V - V_0)\,\theta\,(x/2\sqrt{Kt}),$$

where

$$\theta\,(x) = 2/\sqrt{\pi}\int_0^x e^{-x^2}\,dx.$$

Solving the above for the conditions of positive movement (A), when $x = 1\cdot 25$ cm.,

$t\ = 2$ hours (7200 seconds)
$K = 1\cdot 3 \times 10^{-5}$ (from tables)
$V_0 = 0\cdot 4998$ (percentage chlorides)
$V\ = 0\cdot 5615$ (percentage chlorides)
V_x, the concentration at the spleen fragment,

$= 0\cdot 5000$ per cent. chlorides, or $0\cdot 80$ per cent. sodium chloride.

(The last digit in the values for V_0 and V is unreliable, but the difference between V_0 and V_x due to diffusion is of the right order, subject to the simplifying assumptions made.)

The concentration gradient may be obtained in terms of the difference on two sides of a lymphocyte (say 5 μ diameter) by putting x equal to $1\cdot 2500$ and $1\cdot 2505$ and subtracting. As before, the absolute values are inexact, but the difference is $0\cdot 000001$ per cent. chlorides.

As the above expressions depend on the ratio of the two initial concentrations this difference will be of the same order when the negative movement is obtained (D) with $1\cdot 8$ and $1\cdot 9$ per cent. concentration, and the absolute value of the concentration at x will then be $1\cdot 105$ per cent. chloride, or approximately $1\cdot 8$ per cent. NaCl, the increase only affecting the fourth significant figure.

Conclusions.

The experiments with the diffusion vessel confirm the results and the supposition of a concentration gradient in connection with the tile experiments. It is to be observed that with a similar concentration gradient there is positive movement when the fluid is isotonic, and negative movement when the concentration is sufficiently high to be harmful, an admirable example of the intelligence of the lymphocyte.

On the whole, negative movement was more striking than positive in the diffusion vessel, and a similar observation was made in connection with sodium

chloride in the "tile" preparations. Perhaps every different substance will furnish a different turning point between positive and negative movement when the concentration is continually raised.

I wish to thank Prof. E. M. Wellish for helping me with the mathematical treatment, and Miss W. Mankin for carrying out chloride estimations.

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581 . 143 . 27—1 . 431

The Integration of Plant Behaviour.—II. *The Influence of the Shoot on the Growth of Roots in Seedlings.*

By Sir Frederick Keeble, C.B.E., Sc.D., F.R.S., M. G. Nelson, M.A., and R. Snow, M.A., Fellow of Magdalen College, Oxford.

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Several investigators have already studied the manner in which the growth of roots is affected when the shoots are removed. Kny concluded (1, p. 279) that in young seedlings of *Vicia Faba* and *Zea Mays* the growth in length of the main roots and the increase in weight of the whole root-system were altered very little, if at all, by the removal of the shoot. But Townsend (6, p. 515) found that in seedlings of the same species removal of the shoot distinctly accelerated the growth in length of the root, though not until after a preliminary period of 1 or 2 days, during which the growth of the root was unaltered or slightly retarded. His results certainly appear more convincing than those of Kny.

Pearsall also (4, p. 263) found that in seedlings of *Vicia Faba* removal of the shoot greatly accelerated the increase in volume of the root, the acceleration beginning at some time within the next 2 days. On the other hand it appears

at first sight as if in woody cuttings the removal of buds and shoots produced the opposite effect. For Kny found that in cuttings of *Salix* (1, p. 278) and of *Ampelopsis quinquefolia* (2, p. 616) the continual removal of the developing buds slightly diminished the growth in length of the longest roots, and very greatly diminished the increase in weight of all the roots taken together.

Similarly van der Lek, in a very valuable investigation, found that in various woody cuttings removal of the buds had a very unfavourable influence on the formation and growth of roots (3, p. 215). It therefore appears as if the removal of buds or shoots usually increased the growth of roots in seedlings, but greatly decreased their growth in woody cuttings.

We have repeated the experiment of removing the shoot in young seedlings, and have then measured separately the growth of the main roots and that of the adventitious or secondary roots, as compared with their growth in intact controls. In this way we have obtained results which, as will be explained below, show that the effect of this operation on the growth of roots in seedlings is not so completely different from its effect in woody cuttings as it at first appears to be.

Our experiments were mostly performed on seedlings of *Zea Mays*, which were grown at a constant temperature of 20° C. A few were performed on pea seedlings. All the seedlings were grown in the dark throughout.

Experiment 1.—Ninety seeds of *Zea Mays* were soaked for 24 hours, and then from some the coleoptiles and the young shoots included in them were cut away, while others were left intact. The seeds were sown in damp saw-dust. The main and adventitious roots were measured after 3, 4 and 6 days, and a striking result was disclosed. For after each of these times, it was found that the main roots had indeed grown slightly more in the shootless plants than in the controls, but that the adventitious roots had grown very much less. Thus after 6 days, the mean length of the main roots in 43 shootless plants was 39·5 mm., while in 47 controls it was 37·5 mm., the ratio of the means being 105·3 : 100. But the mean total length of adventitious roots in the shootless plants was only 12·9 mm. per plant, whereas that in the controls was 84·8 mm., the ratio of the means being 15·2 : 100. (By the "total length of adventitious roots" in each plant is meant the sum of the lengths of the several adventitious roots.)

The mean total length of adventitious roots was therefore 6·5 times greater in the controls, and this was partly because they had formed a much greater number of these roots. Thus they formed, on the average 2·2 adventitious roots per plant after 4 days, and 3·2 after 6 days, whereas the shootless plants formed, on the average, only 0·8 adventitious roots after 4 days, and 1·0 after 6 days. The controls therefore had formed 3·2 times more adventitious roots after 6 days, but, besides this, these roots were, on the average, twice as long as in the shootless plants.

Two further experiments were performed, which were similar to the last, except that the shoots were not cut away until 1 or 2 days later. They gave similar results, as follows.

Experiment 2.—The coleoptiles and young shoots were cut away at 24 hours after the time of planting. After a further 3 days, the mean growth of the main roots in 37 shootless plants was 17·40 mm., and in 36 intact controls, 17·06 mm., the ratio being 101·9 : 100. But the mean total length of adventitious roots per plant was, in the shootless plants, 2·63 mm., and in the controls 9·65 mm., the ratio being 27·4 : 100.

The mean number of adventitious roots per plant was, in the shootless plants, 1·10, and the mean length per root was 2·4 mm., while in the controls the mean number was 1·64, and the mean length per root 5·9 mm.

Experiment 3.—The coleoptiles and shoots were cut away at 48 hours after the time of planting.

After a further 4 days, the mean growth of the main roots in 31 shootless plants was 39·82 mm., and in 31 controls 38·09, the ratio being 104·5 : 100.

But the mean total length of adventitious roots was in the shootless plants 2·23 mm., and in the controls 7·54 mm., the ratio being 29·4 : 100.

The mean number of roots in the shootless plants was 2·2 and the mean length per root 1·0 mm., while in the controls the mean number was 3·3 and the mean length per root 2·3 mm.

From the above experiments it can be seen that the removal of the shoot produced two opposite effects. It slightly increased the growth of the main root, but very greatly decreased the formation and growth of adventitious roots. Measurements were also made at shorter periods after the operation in experiments (2) and (3), and these show that the increase in growth of the main root was then rather greater. They are given in the following table :—

Table I.—Mean length of main roots in the shootless seedlings of experiments (2) and (3) at various periods after operation, expressed as a percentage of their length in the controls.

	1 day.	2 days.	3 days.	4 days.
Experiment 2	111·8	103·7	101·9	—
Experiment 3	148·1	115·8	—	104·5

From this table, it is clear that the acceleration of the main roots was greatest during the first day, and then gradually diminished. In Townsend's similar experiment, the acceleration did not diminish for several days. This

may have been because the shoots of his seedlings were longer, and consequently the effect of removing them was greater.

In another experiment (Experiment 4) we soaked seeds of *Zea Mays* for 24 hours, and then removed half of the coleoptile and young shoot from each by a cut passing longitudinally down the middle. The seeds were then planted, and after a further 3 days the length of the adventitious roots on the two sides of the seedlings was measured. It was found that, amongst 24 plants, the mean total length of roots per plant on the side below the remaining half shoot was 2·89 mm., while on the other side it was only 1·81 mm. Moreover the mean total length of roots on both sides together was only 4·70 mm., whereas in 33 controls, with shoots intact, it was 10·27 mm.

Thus removal of half of the coleoptile and shoot considerably diminished the growth of adventitious roots on both sides of the seedlings, but distinctly more so on the side from which the half was removed.

It further seemed desirable to determine what would be the effect of removing the coleoptile only, and leaving the young shoot intact. The following experiment was therefore performed.

Experiment 5.—Pairs of similar young maize seedlings were selected, that had not yet formed adventitious roots, and from one seedling of each pair the coleoptile was removed. The following table shows the mean total length of adventitious roots formed by these plants after a further day or two, expressed as a percentage of their length in the intact controls.

Table II. (Explanation in text.)

	Number of pairs.	24 hours.	48 hours.
Series (1)	7	—	81
Series (2)	8	68	76
Series (3)	9	74	102

From this table, it can be seen that the growth of adventitious roots was usually diminished considerably when the coleoptile alone was removed, but not nearly so much so as when the young shoot also was removed. It appears, therefore, that the greater part of the diminution noted in Experiments 1, 2 and 3 was due to removal of the young shoot.

In order to study directly the effect of removing the young shoot alone, some further experiments were performed upon pea seedlings, as follows:—

Experiment 6.—From a large number of young pea seedlings, 22 pairs were selected whose main roots were found to be of about the same length and to be growing at almost exactly the same rate. When the roots were from 20 to 30 mm. long, the shoots were removed from one seedling of each pair. The seedlings were kept at a constant temperature of 20° C. After a further 22 or 22½ hours, the mean growth of the main roots of the shootless plants was 13·43 mm., while that of the intact controls was 12·43 mm., the ratio being 108 : 100.

Thus in the pea seedlings also removal of the young shoot slightly increased the growth of the main root.

Experiment 7.—Two sets of peas were soaked for 24 hours, and sown in saw-dust. After a further 2 days, the young shoots were removed from some of the seedlings, while an equal number were left intact.

In the first series, consisting of 7 shootless plants and 7 controls, the mean number of lateral roots in the shootless plants after a further 5 days was 8·1, and the mean total length of these roots was 26·0 mm., whereas in the intact controls the mean number was 7·6, and the mean total length 42·9 mm., the ratio of the total lengths being 60 : 100.

In the second series, consisting of five pairs, the mean number of lateral roots in the shootless plants, after 7 days from the operation, was 14, and their mean total length 64·4 mm., whereas in the controls, the mean number was 27 and the mean total length 137·3 mm., the ratio of the total lengths being 46·9 : 100.

Thus in the pea seedlings removal of the young shoot greatly diminished the growth of lateral roots, and in one series the number of lateral roots also, just as in the maize seedlings this operation greatly diminished the number and growth of adventitious roots. In both these species, therefore, removal of the young shoot produces two opposite effects. It slightly increases the growth of the main root, but greatly diminishes the growth of lateral roots, whether these be true secondary roots, as in the pea, or adventitious roots, as in maize.

In regard to each of these effects, the question arises whether it is due to the wound or to the new conditions which arise when the shoot is no longer present. The increase in the growth of the main root was noted by Townsend, who also found that the growth of various parts of the plant was similarly accelerated when injuries were inflicted on them or on other distant parts. These accelerations usually lasted for 4 or 5 days, and they were usually preceded, during the first 24 hours, by a slighter preliminary retardation, which was more pronounced when the injury was more severe. He considered that both these effects were due to an "irritation" set up by the wound and spreading away from it.

It might at first appear more probable that the accelerations were somehow due to the fact that a part of the plant had been removed. But a point in favour of Townsend's view is that he obtained similar accelerations after inflicting wounds that did not remove any part at all, namely, after splitting the root for 10 mm. from a point 5 mm. behind the tip upwards, and after splitting the stem for 10 mm. on one side, near the base (6, pp. 520, 521).

These facts tell against a suggestion made by Pearsall (4, p. 270) that certain of the accelerations noted by Townsend were due to the fact that the parts

removed were no longer drawing on the supply of nutriment. These accelerations should more probably be classed with the increases in rate of respiration in young seedlings, which were found by Richards to last for about 2 days after various injuries, of which one was the splitting of the hypocotyl (5, p. 572). These results he ascribed to a stimulation of some kind.

But whatever may be the explanation of the comparatively slight accelerations of growth after injuries, it seems probable that the large decrease in the growth of lateral roots, which we have noted after removing the young shoot, is a phenomenon of quite a different kind. It seems to be similar to the decrease in the formation of roots in woody cuttings, which follows the removal of their buds or shoots.

An interesting point, noted by van der Lek, tells in favour of this suggestion. He found that certain cuttings of vines, from which the buds had been removed, formed only a small number of roots near the base, and these were only sparingly branched, but extraordinarily thick and stiff. Meanwhile control cuttings on which the buds were left and grew out into shoots, had formed a much larger number of roots which were much more plentifully branched, but much thinner and, to judge from the photographs, rather less long (3, Plates 22 and 23). Thus in these cuttings, just as in the seedlings, it was the amount of branching in the root-system which was diminished by the removal of the buds or shoots.

The reason why this effect was not recorded by Townsend for the seedlings was simply that he measured the main roots only. It is not clear, however, why, in the seedlings, it is only the lateral roots that are retarded by removal of the young shoots, and not the main roots also.

In the experiments with woody cuttings, which lasted for a much longer time, the unfavourable effect of removing buds or shoots is most naturally explained by supposing that these, when present, exert a correspondingly favourable influence on the growth of roots. And since the unfavourable effect of removing the young shoots of seedlings seems to be quite similar, it should probably be explained in the same way. Van der Lek has further been led to consider (3, p. 224) that in woody cuttings the buds or shoots exert their favourable influence by forming a "hormone" which is transported towards the base and stimulates the growth of roots.

Starting from this conclusion, Went (7) has found that, in cuttings of *Acalypha*, expanded leaves increase the formation of roots just as buds do, though less strongly. He has then ingeniously obtained from the cut petioles of these leaves an extract which, when mixed with agar and applied to leafless

and budless cuttings, causes them to form roots. He claims thus to have shown that a substance promoting root-formation is formed in leaves. If this is so, it becomes probable that it is by forming a similar substance that the shoots of young seedlings (or the young leaves of these shoots) increase the formation of lateral roots.

Summary.

1. By the removal of the shoot in young pea seedlings or of the shoot and coleoptile in young maize seedlings, two opposite effects are produced upon the growth of roots. The growth of the main root is slightly increased for a few days, but the growth of secondary or adventitious roots is very greatly decreased.

2. Also by removal of the coleoptile alone in young maize seedlings, the growth of adventitious roots is decreased, but to a much less extent.

3. Reasons are given for considering that the decrease in the growth of lateral roots is similar to the decrease in the formation of roots in woody cuttings which is caused by removal of their buds, and that it is due to the lack of some favourable influence that normally comes from the young shoot.

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612 . 616 . 089 . 843

The Results of Testicular Transplantation in Brown Leghorn Hens.

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The grafting of reproductive tissue in various abnormal environments—auto-, homo-, or heteroplastically—has proved a useful method of approach to the question of the functions and activities of the sex-gland. It has also opened up a phase of this problem which could not be readily examined otherwise—that of the much debated points of the possibility of successful transplantation of reproductive tissue into an individual of the opposite sex, and of the consequence of such transplantation both on the gonads themselves and on their associated sex characters.

Work along these lines has been confined chiefly to mammals. In recent years extensive experiments have been carried out by Steinach, Sand, Lipschütz and Moore. In birds Sand, Pezard, and Zawadowsky have been able to obtain artificial hermaphrodites, but only in isolated cases. The present report concerns a preliminary series of experiments in the fowl, in which testes grafts were implanted into females.

Material and Method.

The fowls used in these experiments were pure bred Brown Leghorns hatched during the period March to July, 1928. The operation consisted of the implantation into a female (whose own reproductive gland had not been disturbed) of both testes from a brother or other chick of approximately the same age. The grafts were implanted in a cavity between the anterior end of the kidney and the ribs (to be referred to as the kidney site). As a rule the left testis was implanted in the left kidney site and the right testis in the right kidney site. A small piece of one or both testes was kept back in a number of cases for implantation subcutaneously on the under surface of the wing; by this means it was hoped to obtain an indication of the behaviour and persistence of the graft. The chicks received their grafts at an age varying from 6–39 days after hatching.

The birds were examined weekly; comb measurements were taken at intervals and a number of feather follicles were marked in the different plumage areas of each individual, from which a succession of feathers was plucked when mature. Fortnightly weighings were made and accurate records of egg production were kept. Twenty of the operated females reached maturity (Table I) but three died shortly afterwards. The cause of death in these cases was not diagnosed.

Table I.

No. of bird.	Age at operation, in days.	No of bird.	Age at operation, in days.
Group A—		Group B—	
42	20	70	19
113	25	618	39
*151	25		
168	13		
169	10	Group C—	
*286	20	445	37
458	17	682	15
462	37		
672	30		
*887	23	Group D—	
904	22	362	13
943	20	735	13
982	21	970	17

* Died during the course of the experiment; observations from them not included in the results.

Results.

It has been found convenient to divide the experimental birds into four groups in order to facilitate description and subsequent discussion. Group A consists of those birds which, during the course of the experiment, showed no deviation in their subsequent history from birds in a control series, except that a few of them exhibited transient modifications of the female type of plumage to that of the male. The other three groups of birds differed from control hens either by the fact that they revealed at *post-mortem* examination persistent testicular grafts, which during the life of the individual had exerted no physiological effect, so far as could be determined from the observations made (Group B), or by their showing profound modification of the primary and secondary functions of the sex gland (Groups C and D). The term "primary function" is used here to denote the elaboration and subsequent discharge of ripe ova, while "secondary function" refers to structural modifications of the accessory sexual apparatus and the external sex dimorphic characters, which

are controlled in their expression or exhibited only in the presence of a physiologically active reproductive gland.

(1) *Comb Growth.*—Since it is recognised that the comb is a secondary sex character in the male and depends for its size and form on the presence of a certain amount of active testis tissue, it was to be expected that in those females which possessed successful testis grafts an effect would be shown in the comb.

In the birds of Groups C and D the combs began to grow noticeably some weeks before those of the control females. Further, they were not typical female combs but resembled the comb of the juvenile male, being thick and upright. At maturity they did not exceed greatly in size (*i.e.*, length and depth) that of a normal laying hen, but their shape, thickness and erect carriage were such that they could only be described as male-type combs. Only two of these birds subsequently laid and in one of these (No. 682), apart from a slight decrease in size, the comb underwent no change following the onset of egg production. In the other bird (No. 445) within a week of laying its first egg the comb had regressed slightly and become lopped in its posterior half. In appearance, however, it was neither a typical male nor a typical female comb.

All the experimental birds comprising Groups A and B developed normal female combs, although at the *post-mortem* examination both the birds in the latter class possessed persistent testis grafts, which in the case of No. 618 revealed some of the testis tubules with the cells in active spermatogenesis.

(2) *Spur Growth.*—Strong spurs are developed by the male Brown Leghorn; in the female they normally remain rudimentary although very infrequently some spur growth may occur in otherwise typical females. It has been shown by the results of castration and ovariotomy in the male and female respectively (Goodale and Domm) that the development of the spurs is inhibited by the reproductive organs in both sexes, but that the ovary inhibits spur growth to a greater extent than the testis.

The experimental birds of Groups A and B possessed only spur rudiments as found in the typical female, but those in Groups C and D exhibited varying grades of spur development. They were least developed in No. 970, where they occurred as large rounded spur buds and reached their greatest development in Nos. 362 and 682. In both these birds the spurs at the conclusion of the experiment were 1 cm. in length. In only one bird were the two spurs firmly attached to the shank bone, in all the other cases either one or both spurs remained loose.

(3) *Plumage.*—In those individuals in which the comb was influenced by

the testis graft (Groups C and D) the plumage also showed marked changes although these were of a temporary nature. In six other birds of Group A (Nos. 42, 151, 458, 904, 943, 982) similar changes appeared but these were much less in extent. The modification in the feathering, which took the form of the appearance of a phase of juvenile male type plumage shortly after the operation, has been dealt with in detail in a previous communication (Greenwood and Blyth); in it the conclusion was arrived at that it indicated a temporary depression or inhibition in the normal course of ovarian function and development.

(4) *Sexual Maturity.*—The age at laying of the first egg in those birds in which the grafts ultimately disappeared (Group A) compared favourably with that of the control birds. In the latter, the average age at first laying was 30 weeks, with a range of variation of from 23 to 39 weeks; in the former it was 33, the limits of variation being 30 and 36 weeks. Of the two birds in Group B which revealed persistent testis grafts at the *post-mortem* examination, one (No. 70) laid at 27 weeks, earlier than any of the experimental birds of Group A, while the other (No. 618), on the other hand, laid later—at 38 weeks.

The two birds in Group C were later in maturing, and No. 445 and No. 682 laid their first egg at 41 and 40 weeks respectively. Moreover they showed further indications of an inhibition of the normal ovarian function in that both these birds became egg-bound in the course of laying their first egg. Cloacal examination showed the mouth of the oviduct to be small and insufficiently dilatable to allow the passage of the egg. The eggs were removed following surgical interference. No difficulty was experienced when subsequent eggs were laid. The birds of Group D (Nos. 362, 735 and 970) are the most interesting of the series in that, although at the present time they are 95, 87 and 84 weeks old respectively, none of them have laid an egg.

An examination of the pelvic bones was made in all operated birds soon after maturity was reached. Birds of Groups A and B, as in the controls, showed that the bones were non-rigid and widely separated. In Nos. 445 and 682, however, these bones were closer together and not quite so pliable, while in the birds of Group D they were hard and firm with only about $\frac{1}{2}$ inch separating the tips of the bones. They were in this respect structurally similar to males.

(5) *Rate of Egg Production and Shape of Eggs.*—It was only in Group C birds that the rate of egg production differed significantly from the controls; whereas in the latter the monthly average for the season was 15·25 eggs, and in the

birds of Groups A and B, 13·3 and 13·7 respectively, in the case of No. 445 it was 10·8 and in No. 682, 4·9.

The shape of a number of eggs (612) from a series of control birds was determined. The shape index (obtained by dividing the length of the egg by its breadth) varied from 1·17 to 1·55 with a mean value of 1·32. Although birds of Group A and B produced normally shaped eggs it was seen that the eggs laid by the two birds of Group C were apparently abnormally long and thin (fig. 1). A determination of the shape indices of eggs laid by these two birds

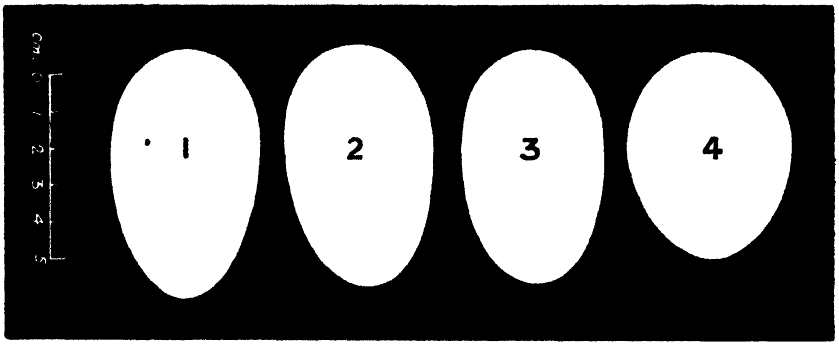

FIG. 1.—Nos. 1–3, eggs laid by hens with testis grafts. No. 4, egg from normal hen.

confirmed these observations, the mean index being found to be 1·46, with a range of variation from 1·33 to 1·70. The individual measurements of eggs laid by controls and Group C birds are graphically shown in fig. 2, and from this it will be seen that a significant modification in egg shape has resulted from the operation in these two birds.

(6) *Fertility.*—The fertility of eggs laid by 31 control hens varied from 68·6 per cent. to 100 per cent. of the eggs set, with an average value of 88·9 per cent. Of the eggs laid by the operated birds, in only one bird, No. 682, was the fertility figure much below the lowest for the control hens, viz., 32·1 per cent. The average fertility of the operated birds (excluding A 682) was 90·1 per cent. No. 445, the other bird in Group C gave a fertility percentage of 91·7.

Before proceeding to suggest possible causes for the lowered fertility in the case of No. 682, it is desirable to state that the system employed was such that the operated birds were divided up among two pens, in each of which a cock was placed. The number of hens to each cock was therefore within the number considered desirable for birds of this breed. No attempts at stud mating were made.

Infertility in this bird could not be related to the fact that the male-type plumage was still exhibited long after maturity had been attained, and that

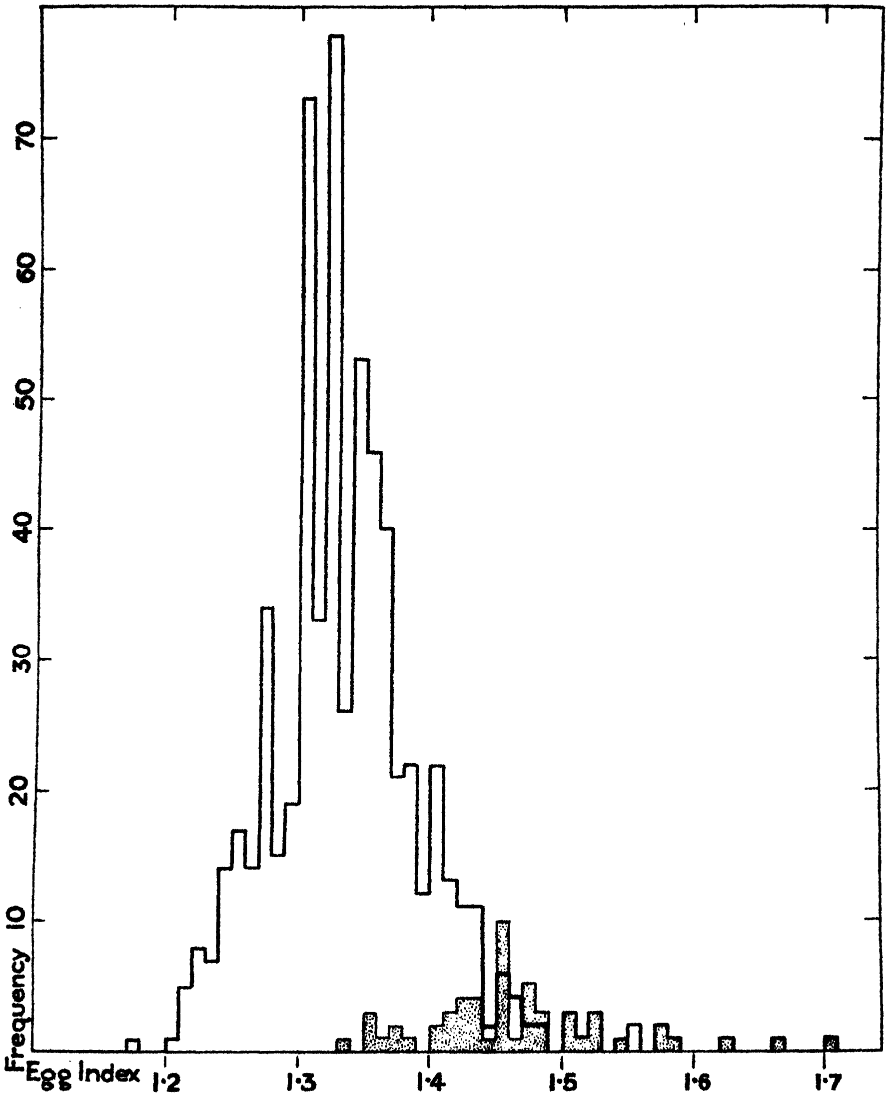

FIG. 2.—Eggs laid by Nos. 445 and 682 indicated by stippling.

this resulted in an antipathy between the male and this bird ; since in the case of No. 445 with similar plumage it may be inferred from the fertility rate that copulation occurred normally. Further, some few of No. 682's eggs proved fertile, indicating that copulation must have taken place. No evidence of the frequency of mating in this bird was secured. There is still another possibility,

infertility may have been due to the direct effect of the graft. McCartney has shown that following the subcutaneous injections of cock's sperm, hen's eggs are infertile for a period of from 12–67 days. It may be that the presence of a testis graft in which spermatogenesis is complete, as indicated by the size of the head furnishings, similarly results in the production of antibodies (or spermatoxins) which interfere with fertility.

(7) *Sex Ratio.*—The number of males per 100 females produced by the control females throughout the year was 114. This figure was obtained from examination of 1000 embryos. Groups A, B and C considered together showed that 106 males per 100 females was produced.

In the two birds from Group C (Nos. 445 and 682), which were profoundly modified in other respects, the actual sex ratio in the former case was 16 males to 14 females. No. 682 throughout a laying period extending from February to October produced at irregular intervals a total of nine fertile eggs ; five of the embryos died during the first 4 days of incubation ; the other four, which lived to an age at which the sex could be determined, were all females.

There was apparently no significant deviation in the sex-ratio of the operated birds when compared with the controls, but the data are too scanty to be of value.

Case Histories.

At the end of the laying period all the birds of Groups A and B were killed and a thorough examination for persistent testis grafts conducted. The birds of Groups C and D, however, because of their manifest interest, have been kept alive to enable further observations to be made upon them. In no case in the birds of Group A was a testis graft found on *post-mortem* examination ; from both the birds of Group B, however, persistent grafts were recovered. Details of these two cases are presented below.

No. 70.—Hatched July 27, 1928. Operated upon when 19 days old and the testes from a brother implanted intra-abdominally and subcutaneously as previously described. This bird from the time of operation until the conclusion of the experiment (when 53 weeks old) differed in no way from a normal hen. The subcutaneous graft became established and persisted until the end of the experiment. About 4 months before death a small piece of the subcutaneous graft was removed and fixed for subsequent histological examination. The *post-mortem* examination revealed a persistent intra-abdominal testis graft on the right side which weighed 0·023 gram. The subcutaneous graft when removed was found to weigh 0·020 gram.

Histology of Testis Grafts.—Both pieces of the subcutaneous graft and the

intra-abdominal graft were similar in histological structure. The grafts consisted of comparatively small testis tubules with a well-defined central lumen. Little evidence of mitotic activity was found in the cells of the germinal epithelium lining the basement membrane of the tubules. The occurrence of the spermatogenetic phase was shown by a relatively few cells in the prophase of the first meiotic division. There was no evidence however of later stages in meiosis. Cell degeneration was confined to a few of the cells in the meiotic phase which were apparently breaking down.

No. 618.—Hatched April 27, 1928. Operated upon when 39 days old and the testes from a brother implanted subcutaneously under the right wing and intra-abdominally on both sides. The bird was killed when 69 weeks old. The wing graft persisted until killed, but as in the case of No. 70, no effect was produced by the graft in this individual, which differed in no way from a normal female, except that the wattles appeared to be longer than those characteristic of the female.

In addition to the subcutaneous graft, two well-developed intra-abdominal grafts, one on either side of the body, were recovered at the *post-mortem* examination. The graft from the right side weighed 0·510 gram, the left graft 0·208 gram, and the subcutaneous graft 0·038 gram.

Histology of Testis Grafts.—The subcutaneous graft was encapsuled by a many-layered fibrous sheath. The tubules were irregular in shape, particularly in the peripheral region of the graft, where they were seen to be long and narrow in section. This appeared to be due to the pressure caused by the enlargement of the central tubules, in which spermatogenesis was taking place. The germinal cells in the meiotic phase were relatively few in number when compared with the normal active testis tubule, and mature spermatozoa were not numerous. Degenerative changes in the tubules were restricted to the sloughing-off of the cells in meiosis into the lumen in some of the tubules.

The right intra-abdominal graft was enclosed by a well developed fibrous capsule. The testis tubules were of two kinds: (1) small tubules situated at the periphery of the graft and in isolated groups throughout the body of the graft. These were similar to the tubules in the testis of the normal male before maturity; and (2) larger tubules in which the spermatogenetic phase was in progress. In some spermatogenesis had just begun, but in the majority of these tubules it was well advanced, as shown by the presence of considerable numbers of mature spermatozoa.

Left Abdominal Graft.—This was similar in histological appearance to the right abdominal graft and differed from it only by reason of the relatively

greater number of tubules (comprising about 50 per cent. of the tissue) similar in structure to the tubules from the normal immature testis. There was very little evidence of degenerative changes in the graft. From the histological examination of the grafts it is assumed that the onset of spermatogenesis had occurred but recently. This view is supported by the following facts: (1) The structure of the graft is similar to that of normal testis soon after the onset of spermatogenesis, in which tubules not yet entering this phase are found alongside enlarged tubules in which spermatogenesis is active; (2) previous studies on testis grafts in different environments have shown that spermatogenesis in such grafts is always related to certain definite and characteristic histological features. The mature sperm produced tend to pass down the tubules and, finding no outlet, collect in certain regions of the graft in large numbers. Degenerative changes are well marked in the tubules and finally give rise to a type of tubule in which only a single layer of cells is found lining the basement membrane, and in these the cytoplasm is fibrillar. None of these features were present in the intra-abdominal grafts described.

It has been shown (Blyth) that, in the male, heterogonic growth in the comb is coincident with spermatogenesis in the testis, and in the case of No. 618 an hypothesis must be developed to account for the fact that in spite of active spermatogenesis in the grafts the comb, though large, was distinctly female in character, although the wattles were somewhat larger than those of a normal female.

It is considered that, in this bird, spermatogenesis has but recently begun and that the necessary stimulus to produce rigidity of the large female comb had not been developed before the conclusion of the experiment. In a previous communication, Greenwood and Crew stated, from observations on a series of experimental birds from which all but small fragments of the testes were removed, that the size of the comb developed was related to the amount of functional activity in the testes fragments and was independent of their weight.

Summary of Results.

At the conclusion of the experiment, from a total of 17 birds, 10 (Group A) showed no evidence of the persistence of the implanted testis tissue, 2 (Group B) possessed persistent testis grafts which were recovered at the *post-mortem* examination, and in the remaining 5 birds (Groups C and D), which have been kept alive, the grafts were still present at the time of writing.

In the birds of Group A the only deviation from normality was the appearance of a transient male phase in the plumage. This was interpreted as a reflection

of a temporary suppression of the ovary following the implantation of the testicular tissue.

Group B birds differed in no other way from normal females, in spite of the fact that grafts were present throughout the life of the individuals. The histological examination of these grafts showed that in one bird (No. 70) the testicular tissue was inactive, while in the other (No. 618) the graft was active and spermatozoa were being produced, though in point of numbers these were considerably below that found in the tubules of the functioning testes of the normal male. The fact that the latter bird possessed a typical female comb may be considered as being due to an insufficient stimulus by the graft for the production of a male-type comb.

It is in the birds of Groups C and D that the effects of successful testis implantation are extremely well marked. In these birds the presence of active testis grafts was shown by the development of male type head furnishings. At maturity a further manifestation of the effect of the grafts was shown by the facts that either (1) the birds did not lay (Group D) or (2) their egg production was below that of normal hens (Group C). In the latter the full development of the accessory sexual apparatus was inhibited—the pelvic bones were insufficiently sprung and the oviduct less well differentiated than in the normal laying hen, since the birds at the laying of the first egg became "eggbound" and the eggs produced were abnormal in shape.

In the females of both these groups aberrant growth of spurs occurred, and in this connection it is interesting to note that "practically all the cases of recorded hermaphroditism, in which there was a suspension of the ovary as an organ of internal secretion, show a more or less pronounced development of spurs" (Domm). These results lead to the conclusion that it is possible, under certain conditions, for persistent testis grafts to inhibit to a varying degree both the primary and the secondary functions of the ovary *in situ*.

Discussion.

From this preliminary study several significant facts have emerged which bear on the problem, much discussed, in recent years, as to how far the reproductive glands of one sex are modified in their development and function by the presence in the same individual of reproductive tissue of the opposite sex.

Steinach, who has studied this question in mammals, has stated that it is difficult, but not impossible, to obtain a graft of a reproductive gland in a normal opposite sexed individual. From this the idea of a "hormonal antago-

nismus " was evolved, it being assumed that the reproductive gland stimulates the homologous and inhibits the heterologous characters.

Shortly afterwards Sand, working with both mammals and fowls, arrived at the conclusion that the antagonism between the gonads and between their actions was not of a specific nature, and explained these relations by his atreptical immunity hypothesis, in which he states—" In every organism are found certain substances which are necessary for the sexual glands, and these substances the latter try to absorb to the greatest possible extent. The normally situated gonads have the best chance of being able to absorb these substances, for which reason heterological (perhaps also homological) gonads transplanted into the organism cannot get enough of these substances and therefore perish."

Lipschütz holds essentially the same view as Steinach, but Moore, on the other hand, states that there is no antagonism between the gonads, and has adduced facts to show that not only did the females bearing testis grafts remain normal in all their secondary sex characters—ovary, genital passages pysche, etc.—but they actually reproduced. He states further that spermatogenesis continues in the testis graft in the pregnant female, but is never complete. The results of his experiments with rats lead him to conclude that there is no justification for either the " antagonism " or the " atreptical immunity " hypothesis.

These previous workers have, like the present writers, come to the conclusion that it is possible to obtain grafts of reproductive tissue in normal individuals of the opposite sex, but they disagree as to the ease with which these grafts " take." The number of experimental individuals in the present series is insufficient to yield definite conclusions on this latter point and it is considered preferable to postpone the discussion of the question until such time as data are forthcoming from a further series of experiments now under way, in which more than 100 testis engrafted females are included. But if the marked modifications in reproductive function in these fowls with persistent testis grafts are considered, it is obvious that it is impossible to agree with Moore that there is no antagonism between the gonads. It remains to define if possible the nature of this interference between the two types of reproductive tissue.

From the gradations of sex characters displayed by the different individuals of the series it is clear that one is not dealing here with a complete dominance of one type of gonad over the other, for if such had been the case all the birds would have exhibited one set of sex characters only. In the two birds of Group

B the development and activity of the ovary have apparently run their normal course, while the testes grafts either remained relatively immature, showing only early stages in spermatogenesis (No. 70) or revealed a subnormal rate of spermatozoon-production (No. 682), suggesting that a partial suppression of the graft by the normally active gonad *in situ* may occur. However, in the other extreme class (Group D), the testis is apparently in full activity (as judged by the size of the comb), but the ovary while inhibiting male plumage is incapable of its primary function, egg-production. From this it is clear that it is not a question of a partial dominance of the gonad of the host over the graft, or of ovary over testes, but that the antipathy is mutual. It may indeed be that, in this latter group, we are dealing with conditions exactly reciprocal to those obtaining in Group B, in that the testes may be functioning normally and the ovary sub-normally. In the absence of histological evidence, however, we are not justified in assuming this, since although the comb is of the male type it is known that the presence of only a relatively small amount of active testicular tissue is necessary for the development and maintenance of such head furnishings

The birds of Group C yield an intermediate stage between the two classes discussed above; in these, both ovary and testes grafts are active, in so far as their primary functions are concerned, yet the diminished rate of egg-production and incomplete development of the accessory sex apparatus, on the one hand, and the lopping and decrease in size of No. 445's comb, on the other, indicate that neither type of gonad is fully active.

Thus it appears that while it is not possible for both types of gonad to function to their fullest extent in one and the same individual, yet we can have a coincident partial development of both, with a consequent partial development of function. Further, arguing from Group B, it appears that we may even have a complete development and function of one type of tissue combined with a very subnormal grade of differentiation in that of the opposite sexed tissue.

These findings, then, with regard to the relations of the gonads themselves, are not in opposition either to the "antagonism" hypothesis of Steinach or to the "atreptical immunity" theory of Sand. Moore, as has already been stated, has come to the conclusion that there is no antagonism between the gonads, but consideration of his experimental data by no means proves this contention.

He has argued that, since the implantation of two testes grafts in a normal female rodent had no effect on maturation, ovulation, pregnancy or the state

of the uterine tubes, the heterologous characters are not inhibited by the grafts. Since, however, in these grafts, spermatogenesis continues, but is never complete, his successfully engrafted individuals are, in our opinion, comparable with the bird, No. 70, in Group B, and provide only negative evidence of a lack of antagonism between the gonads. In view of our findings it is evident that before such an antagonism could be disproved it was necessary to show that, when both gonads are present in one and the same individual, it is possible for each of them to perform their *primary* and *secondary* functions in a normal manner. But his extensive studies of experimental cryptorchidism have proved that only in its normal position—the scrotum—can mammalian testis complete the process of spermatogenesis. Therefore it is impossible, in the female rodents he used, to obtain testes grafts sufficiently active to yield positive proof of his contention. That he has proved the testes grafts to be as active as they can be outside the scrotum does not alter the fact that their spermatogenetic activity was subnormal. Thus so far neither the hypothesis of Steinach nor that of Sand has been disproved.

The observations on comb development in the birds with persistent grafts throw some light on the question of whether the apparent antagonism in the gonadic activity arises from a mutual neutralisation of the influence of the respective gonads or from a balance of their functioning. In the former case a tendency to the capon type is to be expected ; in the latter, where both gonads are acting in the same direction, a comb *no less* in size than that of the normal female should be present. This is what was found.

The idea is further substantiated if we consider the two laying birds (Nos. 445 and 682) in which the secondary sex functions of the ovary were upset. In these not only were the oviduct and pelvic ligaments insufficiently prepared for laying, but the primary function itself was disturbed, in that egg production was subnormal—indicating that a lowered functioning of the ovary was the factor responsible for the incomplete development of the female sex characters, and not the inhibiting activity of the testis. Still further proof that we are not dealing here with a hormonal antagonism, such as Steinach and Lipschütz have postulated, is presented by No. 682 and the birds of Group D ; in these, when adult, a typically male comb was accompanied by normal female plumage.

At the present time only the hypothesis of Sand—that of a specific foodstuff necessary for the maintenance and function of reproductive tissue and present only in limited amounts—can accommodate our findings of a balance between the activities of the gonads, and provide a reasonable explanation of the gradation of sex characters found in the experimental birds when adult, and

also of the temporary male phase in plumage exhibited soon after the implantation of the graft. In the case of the latter phenomenon it is easy to see that where the testes grafts became vascularised the sudden increased demand on the specific foodstuff (whatever it may be—endocrine or otherwise) would have the effect of decreasing the limited amount normally supplied to the developing ovary, thus causing a temporary suppression of its function.

Summary.

In a series of normal hens it has been found possible to obtain persistent grafts of testicular substance.

Modifications in the functions of the ovary have been produced, both in regard to the production of eggs and to the development of the secondary sex characters.

The bearing of these results on the divergent theories of Steinach, Sand and Moore with regard to gonad interaction and function is discussed.

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Studies on the Rhone Glacier, 1927.—*The Structure of the Ice in a Compressed Zone on the South-Eastern Part of the Glacier.*

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(Communicated by Prof. W. W. Watts, F.R.S.—Received January 25, 1930.)

[Plates 19-21.]

1. *Introduction; General Questions Involved.*

The following paper embodies the results of a study of the Rhone Glacier in the summer of 1927. The object of the investigation was to map the structures developed in a selected zone which had been subjected to the influence of concentrated pressure. In the Alpine area this problem has been overshadowed by investigation of the wider problems of the physics of a glacier as a whole, as evidenced by the large amount of research accomplished, notably on the viscosity, the granular structure, and the movement of the ice (9 and 12). Deeley, for example, discussing the genesis of movement of a glacier, writes as follows (4) :—

> "Throughout its course a glacier is urged along, not by a pressure from above, but by gravity acting upon each molecule of ice during its whole course . . . No doubt *thrust* does play some part in the movement . . . but it is quite a subordinate feature and only produces local effects" (1895).

The point of view of a geologist regarding ice-motion has been given by Lamplugh, who in 1903 wrote as follows (6) :—

> "The much discussed problem as to the physics of ice motion is not of immediate consequence to the field-worker in glacial geology, who sees all round him the evidence that the moving mass behaved to all intents and purposes as a plastic body, be the cause what it may."

But to the student of disturbed Pleistocene Drift deposits the behaviour of ice in a zone of compression is of great importance. Up to the latter part of the 19th century information on this subject was largely based on inferences derived from the morphological study of structures in disturbed areas of glacial drift, such as Cromer (2), Møen in Denmark (14), and Rügen in Germany (7). Clement Reid, for example, speaking of the Norfolk Contorted Drift deposits, came to the following conclusion (1882) (2) :—

> "The extent of the disturbance at Trimingham also points to the employment of enormous force, as well as steady pressure. Though much has yet to be explained as to the cause of the flow of an ice-sheet, the acceptation of this agency seems the only way of accounting for these phenomena."

The demand of geologists for enormous force to account for the phenomena was consistently opposed by physicists, as being alien to the *modus operandi* of ice action. Towards the end of the 19th century, fresh light was thrown on the problem of the behaviour of ice under compression, as a result of investigations by American geologists in Greenland (3), and of later work in Spitsbergen by Norwegian and British geologists (5 and 11). The principles elicited by their work are comparatively simple :—Obstruction to movement of a glacier results in the formation of a zone of compression. The ice seeks relief from this pressure (1) by the development of thrust-planes associated with upward movement, and (2) by a second movement, at right angles to the main direction of pressure, caused by tension. These movements are betrayed in Arctic glaciers by the englacial material incorporated in them, which acquires and reveals the structures of the ice.

Such structures are strikingly analogous to those exhibited by disturbed Pleistocene Drift deposits ; as, for instance, those of the Ipswich district ; and these may therefore be regarded as glacial pseudo-morphs (13). They are not the result of "enormous" ice-sheet pressure brought to bear on flat-lying deposits, previously laid down, but material carried on and, in a glacier, contorted and twisted by its movement, imbricated by the thrust-planes formed on the ice-ward slope of obstructions to ice-movement, and finally laid down without much re-arrangement and without loss of its structure when the ice melted. Neither are the great masses of chalk and other rocks included in the drift due to shifting of such material in mass from its outcrop, but to the moulding by ice of separate lenticles of country-rock.

Thus the evidence of the drift forms a supplement and sequel to that derived from existing (Arctic and Antarctic) glaciers, the geologist being able to study the final results of varying processes operating in moving viscous bodies over long periods of time. It, therefore, becomes necessary to study in more detail the physics of compressed ice in Alpine glaciers, and for this purpose the writer selected a section of the Rhone Glacier. The detailed trigonometrical work accomplished by Swiss workers since 1874 in this glacier has provided a mass of data on the differential movement of ice, which has furnished an

admirable starting point for the elucidation of the special problem now dealt with.

II. *The South-eastern Embayment of the Rhone Glacier.*

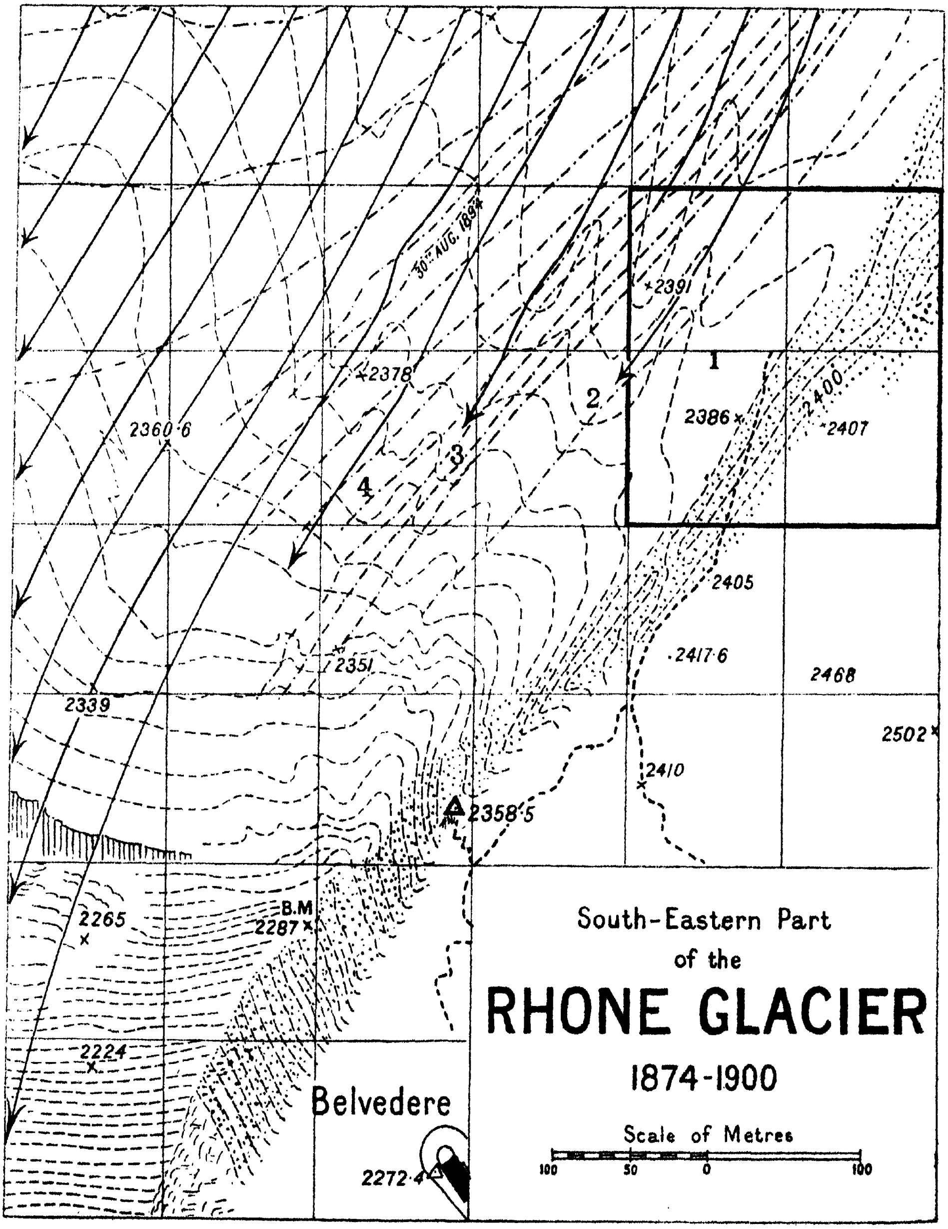
The area selected for study was the south-eastern concave flank of the Rhone Glacier, adjacent to the termination of the path which leads to the Fort and the road to the Furka Pass, and at a distance of about 500 metres north-east of the Belvedere Hotel (see accompanying map, fig. 1). The reason for regarding this area as a zone of compression is as follows :—

The ice to the north of it, for a considerable distance, showed a regular banded structure, with a normal trend more or less parallel to the side of the glacier (Plate 19, A). But inside the area itself the ice was broken (by fissures) suggesting planes of movement, and within each of the separate units thus severed the banding was independent and without relationship to that of its neighbours (Plate 19, B and C).

In addition, the surface of the ice showed special features, four longitudinal ridges being separated from one another by elliptical basins, each having a specialised series of crevasses (Plate 20, A, B, C, D). The well-marked series of marginal crevasses were, on the other hand, clearly associated with the movement of the glacier as a whole. Some of the crevasses extended into the eastern elliptical basin. Vertical sections in the lateral walls of the ridges showed highly inclined thrust-planes, associated with contorted ice (fig. 3).

Similar ridges have been described by Forbes (1). At a point of the Mer de Glace named *l'Angle*, where the glacier was about to turn at a considerable angle with its former direction, Forbes noted in 1844 compact ridges parallel to one another, and separated by localised systems of crevasses. These ridges were characteristic of this part of the glacier. " The crevasses may be nearly transverse to the glacier, whilst the *systems* of crevasses form an angle of perhaps 30° with the transverse line. The veined structure again cuts the crevasses at right angles." (p. 205). . . . " I have found a repetition of this phenomenon of a series of discontinuous but parallel fissures ranged along a line or axis oblique to their direction, to recur at several points where the strain is very violent " (p. 205). . . . " where the violence of the pressure opens a system of such fissures to relieve it, the bands, or systems of surfaces of molecular discontinuity, disappear, or are less well developed " (p. 205).

Before describing the structure of the compressed area the evidence of the movement of the glacier as a whole will be considered in its bearing on the present problem.



- - - - - - - - - Contours.

·—·—·—·—· Successive margins of advancing ice.

————→ Direction of movement of bench-marks ((*repères*).

FIG. 1. 1, 2, 3, 4 indicates ridges of ice. The thick square shows area described. This map is a portion of Plan No. 3 (with omissions) of the Swiss Glacier Commission [10]. Reproduced by permission of the Glacier Committee of the Société Helvétique des Sciences Naturelles and Prof. Dr. P. L. Mercanton.

III. *Trends of Movement on the Rhone Glacier.*

The lines of movement of the Rhone Glacier are approximately shown on the map, fig. 2, which embodies the results of the Swiss Glacier Commission (1874–1900) (10). The lines of movement may be divided into three groups as follows :—

1. Those in the *upper* part which converge.
2. Those in the *intermediate* part which tend to parallelism with one another and the side.
3. Those in the *lower* part which diverge.

It is the intermediate part, 2, on which the present paper has a direct bearing. These lines for convenience may be sub-divided into three groups (*a*), (*b*) and (*c*) (see fig. 2) as follows :—

(*a*) A central group of curved lines which pass completely down the glacier and ultimately reach its termination.

(*b*) A marginal group having an unusual orientation. These lines bend gradually towards the nearest flank and terminate there at an angle more or less acute.

(*c*) A third group, on the eastern side of the glacier, intermediate between (*a*) and (*b*). These lines are marked only partially, and terminate in the south-eastern area as shown in figs. 1 and 2.

Speaking of the loss of *repères*, Mercanton writes as follows (10, pp. 154–5) :—

"Malheureusement la disparition des repères marginaux, les plus intéressants, nous a privé trop tôt d'un élément d'information de très grand prix. Ce que nous savons suffit encore à confirmer les vues de Finsterwalder : dans le collecteur, les lignes de mouvement se rapprochent de l'axe d'écoulement du glacier en tendant au parallèlisme."

The reason for the loss of the *repères* will be dealt with at a later stage in this paper.

The ice between that traversed by the central group of lines (*a*) and the eastern margin of the glacier, forms a segment of a circle, in which the movement lines are either directed towards the sides, or lost as stated above. How does this ice ultimately reach the end of the glacier? Only two alternatives seem possible :—

1. The lines or movement may converge : or
2. The movement of the ice within the segment may be of a specialised type.

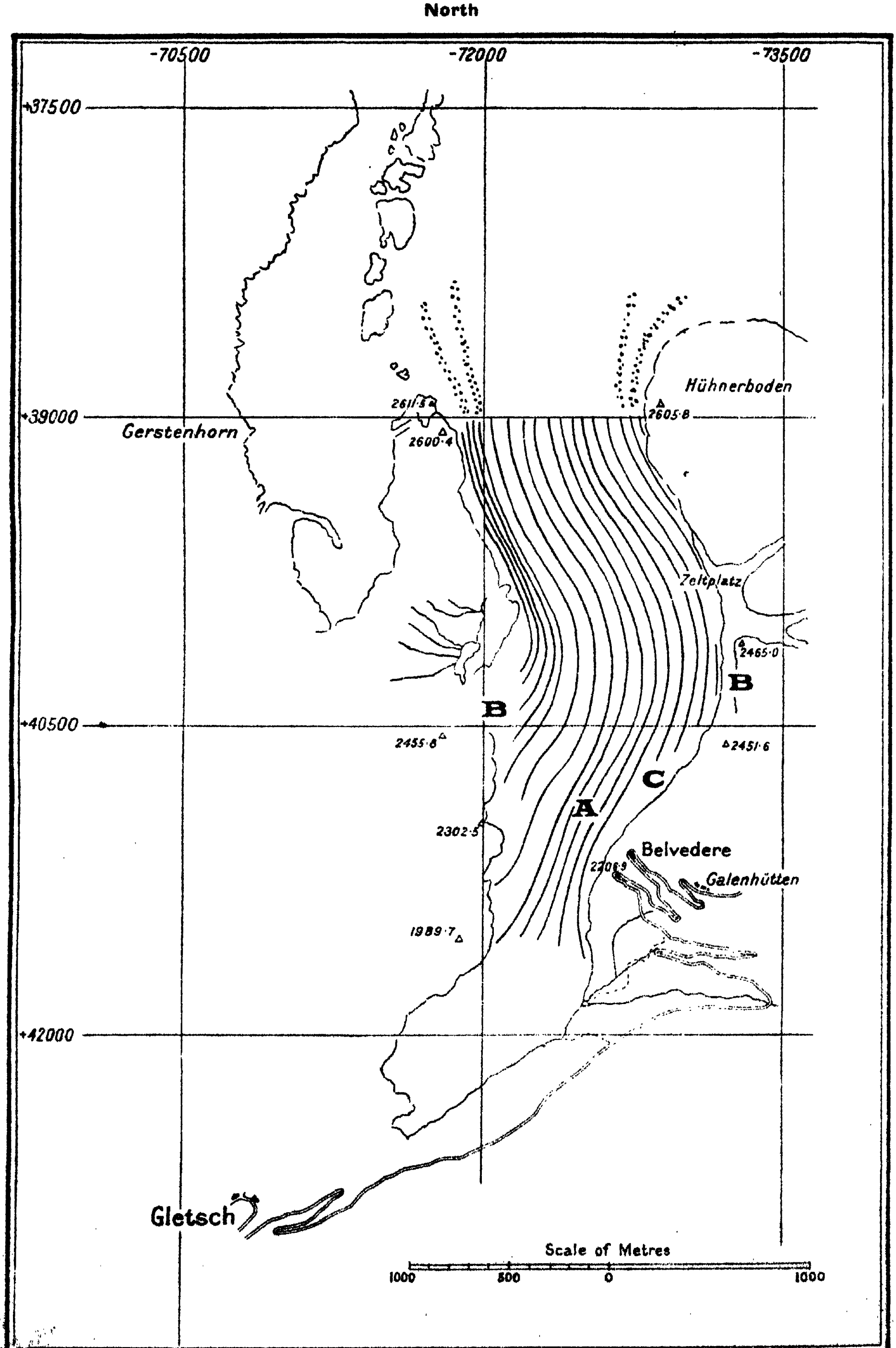


FIG. 2.—Rhone Glacier, 1916. Based on plans No. 1 and No. 3 of the Swiss Glacier Commission [10]. Reproduced by permission.

On this problem it is well to recall the pioneer observations of Forbes, recorded in his 'Theory of Glaciers' (1). Describing, with a diagram, an embayment of the glacier of La Brenva, 1842, he writes :—

> "It seems clear that all the ice within the line FED [that is, the ice in the more central part of the glacier], or between it and the shore is *embayed*, as it were, and has but little motion in consequence of the intense pressure with which the whole mass of the glacier is urged against the side of the valley" (pp. 182–3).

Forbes's trigonometrical measurements confirmed this view, for whereas the outer zone of ice FED moved 14·2 inches in 24 hours, the embayed ice was practically stationary relative to the general movement of the glacier, but had moved outwards and upwards against the margin of the glacier by 2 inches. Forbes compared the behaviour of the embayed ice to the rotatory motion of water under similar circumstances (pp. 182–6).

The structure of the south-eastern embayment of the Rhone Glacier confirmed the view that movement in that area was of a specialised type. The ice in the segment of the glacier moving downwards, collected in the south-eastern embayment, and produced a zone of ice under concentrated pressure.

IV. *Method of Study.*

The trigonometrical survey of glaciers has resulted in the accumulation o a mass of data on the differential movement and structure of the ice. In spite of this excellent work, however, the scale of such maps is inadequate to show the detailed structure, and further, in no case, to my knowledge, has a map of a glacier been published which shows the thrust-planes developed more especially in compressed areas.

Results previously obtained by detailed mapping of disturbed drift deposits of the Ipswich district (13), encouraged the author to adopt the same method on the Rhone Glacier. The method adopted was the usual one employed by geologists, viz., the detailed mapping of the structures displayed, and the recording of vertical sections where visible. The surface of the ice was marked out in squares, 50 feet on the side, the sides being orientated N.-S., E.-W. (magnetic).

The square was outlined by two 100-feet tape-lines, and the structure seen on the ice plotted to a scale of 50 feet to an inch. On the completion of one square an adjoining one was marked out and the structure recorded as before.

The total number of squares completed was about 70, representing a total area of about 4 acres. The results are plotted on the plan (Plate 21).

V. *Structure of the Ice* (Plate 21, map).

The two most striking features of the structure of the south-eastern embayment of the Rhone Glacier were (1) the presence of compressed blocks of ice, and (2) the association of these blocks with thrust-planes.

(1) *Compressed Block of Ice* (Plate 19, *cf.*, A and B).—The banded structure of the ice had suffered extreme dislocation, and was orientated in many directions in the separate blocks. The ice was frequently contorted, and the bands of the ice in each block usually curved near the margins. The blocks of ice were of various shapes but were usually triangular in form. Towards the southern part of the map (Plate 21) it will be noticed that the ice was compressed and contorted into narrow strips. Such a structure must inevitably mean mass-movement due to pressure, and indicates a type of movement unlike that already described by Deeley.

(2) *Thrust-planes* (Plate 19, C).—The planes separating the compressed blocks (1) were thrust-planes. Their orientation was significant, for they were arranged in fan-shaped groups, each group radiating from a point usually, but not always, adjacent to the easterly margin of the glacier. The orientation of the thrust-planes in each group varied from approximately north-west to south-east, to nearly east and west when traced down the glacier. The latter direction, for example, was dominant in the southern part, as shown in the map (Plate 21).

The association of the thrust-planes with the compressed blocks showed that the latter occurred between the "jaws" of thrust-planes. Pressure of ice between the thrust-planes had apparently squeezed the blocks away from the lateral margin of the glacier and towards the adjacent elliptical basin, which, as will be shown later, was a zone of tension.

The arrangement of the thrust-planes clearly suggested differential movement of the ice, that near the margin moving more slowly than the ice farther away from the side. The orientation of the thrust-planes also supported the view of a progressive change in direction of movement down the glacier. The movement towards the south-west, for example, had been slowly deflected to a movement more nearly southwards.

Vertical sections in the walls of the elliptical basins were extremely instructive. These sections showed a series of highly inclined thrust-planes associated with

contorted ice. The sections proved that the ridges of ice were due to an upward movement along thrust-planes (fig. 3).

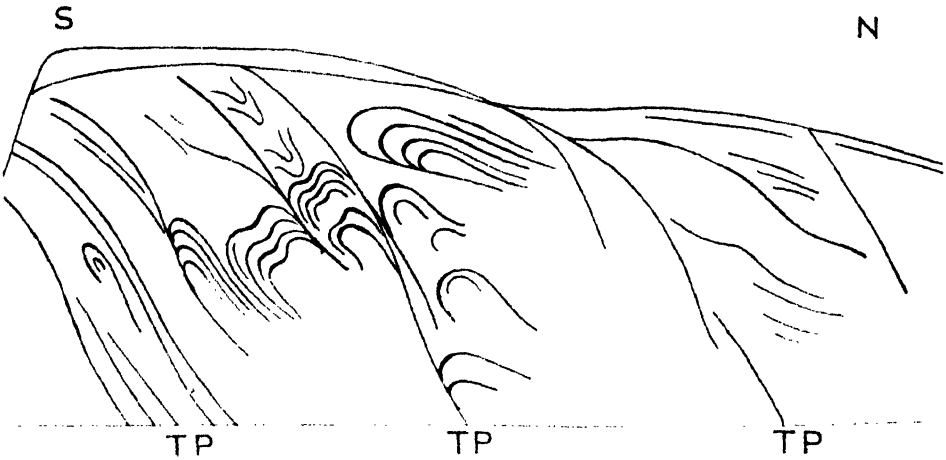


FIG. 3.—Vertical section in the side of one of the elliptical basins. Contorted ice associated with thrust-planes (T.P.). Height of section about 40 feet.

The amount of detailed work which had to be done only allowed the structure to be recorded in the area adjacent to the most easterly ridge, but the principles formulated from this area will apply to the adjacent ridges.

VI. *Relationship between General Movement of Rhone Glacier and that of the Compressed Area.*

The lines of movement of the lower part of the Rhone Glacier (fig. 2) indicate a general direction of movement towards the south-west. The thrust-planes in the compressed area, on the other hand, showed this trend to have been deflected to a movement to the south. Such a deflection was evidently due to the retarding by friction of the ice adjacent to the flank, against the side of the glacier.

The point where deflection of movement became apparent was a tensional area, for the ice at this point was drawn apart. This area was marked by the easterly elliptical basin, the floor of which gradually rose towards the south where the southerly movement alone became operative (Plate 20, A).

The general effect of these movements was reflected in the structure. The compressed ice had obtained relief in two ways, (1) by an upward rise, thus forming the ridge (section, fig. 3); (2) by lateral squeezing towards the elliptical basin (see map, Plate 21).

The zone of greatest compression occurred adjacent to the projecting spur of the side of the glacier south of the map. These observations suggested that the loss of bench-marks mentioned by Mercanton was due to specialised types of movement of the ice. These movements would raise the bench-marks to higher levels and deflect them towards the more central part of the glacier. Hence parallelism of the lines of movement would, as a natural consequence, be destroyed.

The sequence of events which led to the formation of ridge 1 and its elliptical basin (Plate 20, A) was repeated at a somewhat later stage in the more central parts of the glacier, resulting in the progressive formation of ridges 2, 3 and 4, and their associated basins (map, fig. 1).

VII. *Application of the Principles of the Structure of the Compressed Zone of the Rhone Glacier to Areas of Disturbed Pleistocene Drift Deposits.*

As already mentioned in the Introduction, the value to geologists of work on the structure of compressed areas of " living " glaciers lies in the fact that similar structures are preserved in disturbed drift deposits, owing to the removal of the interstitial ice associated with englacial material. Amongst the structures preserved, flow-curves and thrust-planes are dominant.

The groups of radiating thrust-planes seen on the surface of the Rhone Glacier are the same in principle as those also seen, in plan, in the disturbed deposits of the Hadleigh Road Area, Ipswich (13). In the Rügen area the lines of trend of the thrust-planes have been mapped by Keilhack (8). As a result of the present investigation it occurred to the author to extend these coastal lines seawards by means of dotted lines. The result of so doing was to show that the thrust-planes arranged themselves into a series of groups. The thrust-planes of each group converged towards a common point, whilst there was a progressive deflection of the dominant trend of each group, when compared with its neighbour to the south (taf. IV, fig. 1 (16)). The total distance in which this phenomenon could be traced in the Rügen area was 5 miles.

In explanation of this extensive zone of compression, it must be remembered that the structure displayed in the cliffs represents ice-action on a regional scale. The compressed zone is therefore quite a local feature compared with the probable area of the Baltic Glacier. The repetition seen in the Rügen cliffs demonstrates in a convincing manner imbricate-structure (taf. V (16)). The lower boulder clay, which is divided repeatedly into isolated strips, forms an agreed horizon, all workers agreeing that it once formed a connected stratum

of boulder-clay. In the absence of englacial material in the Rhone Glacier, it is difficult, perhaps impossible, to prove a similar repetition of structures.

VIII. *Summary.*

The investigation of the Rhone Glacier by the author in 1927 was confined to two lines of enquiry : (1) the structure of the ice forming the concave side of the south-eastern flank of the glacier ; (2) the relationship between the air-temperature and rate of the surface melting of the ice.

(1) The surface of the marginal ice was marked into 50-foot squares, and the structure plotted on a map to a scale of 50 feet to an inch. The ice formed a mound dissected into ridges by three longitudinal, basin-shaped trenches, which were heavily crevassed laterally and bounded by crevasse-like walls of ice longitudinally. Thrust-planes dipping at high angles formed a characteristic feature of the ridges. In plan they formed radiating groups, the fulcrum of each group being near the margin of the lateral moraine. The trend of these thrust-planes varied progressively southwards, from N.W.-S.E. to E.-W. approximately, and adjacent pairs formed the jaws of squeezed wedges of ice showing displaced ribbon-structure. Crevasses radiated from the lateral moraine and in places dissected the junctions of the thrust-planes. The structure as a whole suggests pivotal movement of the compressed marginal ice, the "trenches" representing tensional areas due to the deviation in direction southwards of the movement of the ice, from the normal south-westerly trend. Relief from pressure was obtained both laterally and longitudinally by the squeezing inwards of the ice towards the tensional areas on the one hand, and by the upward rising of the ice along thrust-planes on the other.

The second line of enquiry (2) forms the subject of another paper published by the Royal Meteorological Society, London (17).

IX. *Conclusion.*

In conclusion the author expresses his thanks to Prof. P. L. Mercanton and the Commission of Glaciers (S.H.S.N.) for permission to reproduce the maps shown in figs. 1 and 2. To Prof. W. W. Watts, F.R.S., he is indebted for granting facilities for the working out of the results in the Geological Department of the Imperial College of Science and Technology, London. The expenses of this investigation were defrayed by a Government grant from the Royal Society, for which the author tenders his thanks.

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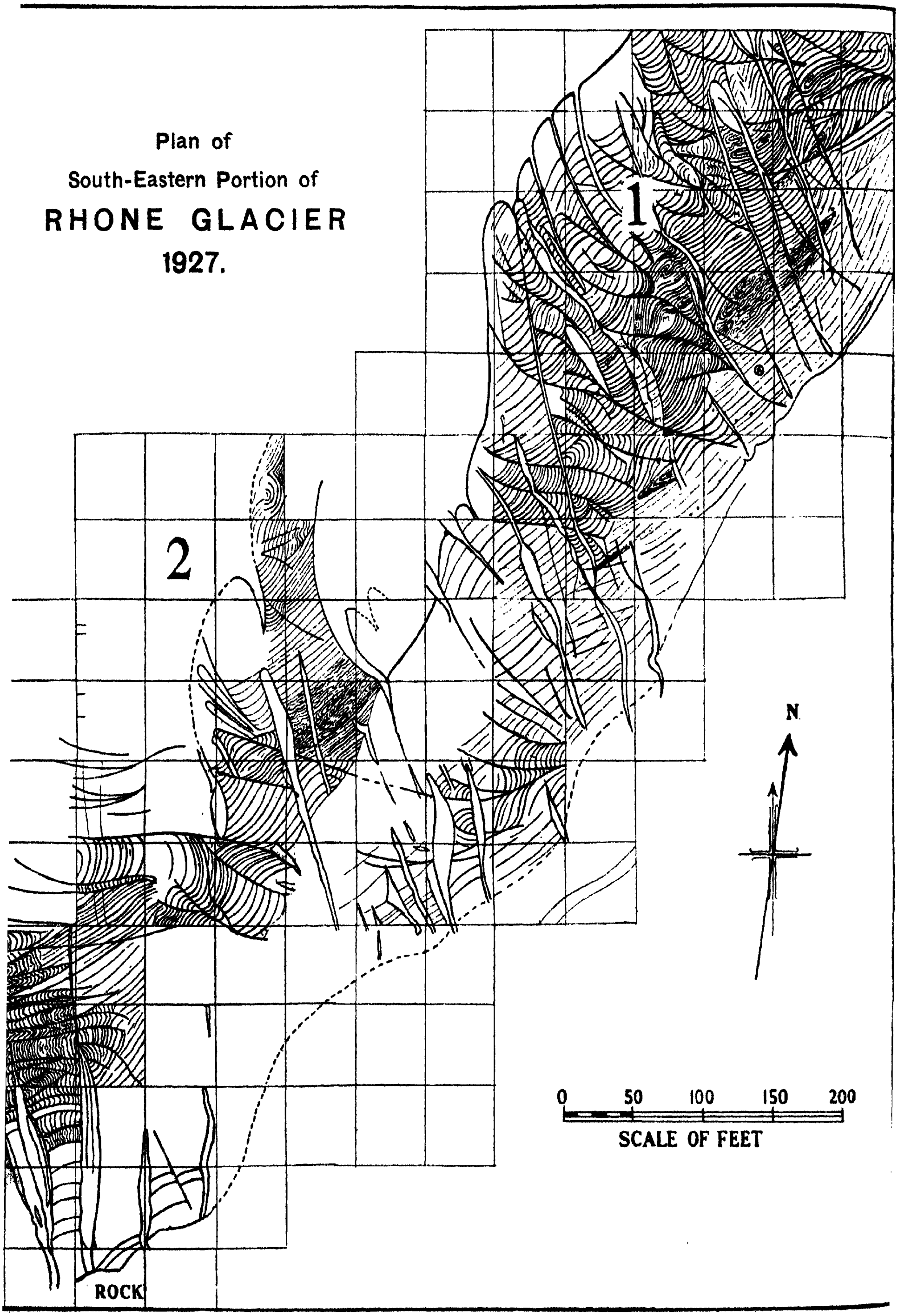
B

C

A

(*Facing p.* 214.)


Plan of
South-Eastern Portion of
RHONE GLACIER
1927.
1
2
N
0 50 100 150 200
SCALE OF FEET
ROCK

DESCRIPTION OF PLATES 19–21.

PLATE 19.

Structure of Ice in S.E. portion of Rhone Glacier, 1927.

A.—Normal arrangement of ribbon-structure in the ice, dissected by a crevasse. B.—Abnormal arrangement of ribbon-structure in the ice. One of the compressed blocks of ice associated with thrust-planes. C.—Middle portion of the Rhone Glacier. The compressed zone occurs in the foreground and displays thrust-planes, with ice raised to higher levels.

PLATE 20.

Ridges and Basins in S.E. part of Rhone Glacier, 1927.

A.—Easterly ridge of ice (ridge 1 of map, fig. 1), with associated elliptical basin and portion of ridge 2. B.—Ridge 2 of map, fig. 1. C.—Upper portion of crevassed basin of A. D.—Portion of the same basin as C, showing the steep easterly wall.

PLATE 21.

Plan of S.E. portion of Glacier.

Firm lines = thrust-planes. Open spaces between firm lines = crevasses. Narrow parallel lines = ribbon structures. Blank squares in southern part = snow-covered areas. In upper part of map blank squares represent unmapped areas.

1 = Ridge 1 of map, fig. 1, p. 206, and Plate 20, A.
2 = Ridge 2 of map, fig. 1, p. 206, and Plate 20, B.

A crevassed basin occurs between 1 and 2.

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Structure in very Permeable Collodion Gel Films and its Significance in Filtration Problems.

By William Joseph Elford, National Institute for Medical Research.

(Communicated by J. E. Barnard, F.R.S.—Received January 31, 1930.)

(Plate 22.)

The problem of gel structure is one of general scientific interest and importance, but it is in regard more particularly to its significance in ultra-filtration that the present work has to deal. The observations concern collodion gel films, and have been made during an investigation into the problem of filtration and filterable viruses, which has involved a close study of the behaviour of various types of gel membranes employed for ultra-filtration purposes.

The most widely used membranes are made from collodion, a solution of nitro-cellulose in some suitable solvent, like acetic acid or ether/alcohol mixture. Two methods are available for preparing such membranes, according as to whether the solvent is volatile, like ether/alcohol, or non-volatile like acetic acid. In the former case the collodion is spread in a uniform layer over a glass or mercury surface and the solvents allowed to evaporate under standard conditions until the film just "sets," *i.e.*, incipient gelation occurs. The remaining solvent is then washed out by immersing the film in water, which completes the gelling process. The alternative method, used when employing acetic acid collodion, consists in impregnating filter-paper (which serves to support the delicate film) with the collodion and then washing in water to replace the solvent acetic acid, and so gel the collodion.

At first sight there would appear to be little difference in the actual methods of obtaining the gel film, since the essential process in each is the replacement of solvent by the non-solvent water. However, a closer study reveals a difference which is fundamentally important in determining the resultant structure. Whereas in the case of ether/alcohol collodion membranes incipient gelation is allowed to set in prior to washing out the remaining solvent with water, which procedure merely fixes the potentially existent structure, there is clearly a different process in the case of glacial acetic acid collodion membranes. Here the collodion at the time of gelling in water is not in the incipient gel state, but exists as a viscous solution, the viscosity being a function of the nitrocellulose

concentration. Now when immersed in water acetic acid diffuses out into the main bulk of the water, as the latter slowly diffuses into the collodion film, the progressive replacement of solvent by non-solvent resulting in desolvation and coagulation of the colloidal nitro-cellulose aggregates. Whether this coagulating process is complete, yielding a fibrous precipitate, or only partially so, yielding a gel, depends entirely upon the relative bulk and distribution of the participitating reactants, nitro-cellulose aggregates, acetic acid and water. The process is dependent upon the rate of inter-diffusion, which is in general slow, falling off from an initial maximum value ; hence differences in structure between very thin and thick films are to be expected. This has actually been found to be the case, as will be pointed out later.

The two types of film exhibit marked differences in structure, which affords a complete explanation of the dissimilarities to be noted in their general properties. These properties are summarised in the following Table I.

Table I.

Property.	Acetic acid collodion membranes. Microgel films.	Ether/alcohol collodion membranes. Ultragel films.
Permeability	Order of 1–5 μ downwards. Can be prepared readily permeable to ordinary bacteria like *B. coli*	100 $\mu\mu$ downwards. Proteins and finer colloids pass,—coarser suspensions retained.
Standard	Good reproducibility as indicated by rate of flow of water—within 10 per cent.	Excellent reproducibility. R.F.W. $<$ 1 per cent.
Limits of pore size	Pore size varies over comparatively wide range, but within fairly well defined limits	Pore size relatively uniform.
Distribution of pores	Fairly regular	Very uniform.
Strength	Fragile to moderate	Moderate to strong.

In a systematic study of the filtration capabilities of acetic acid collodion membranes it was pointed out by the author* that since certain of these membranes of average pore size, 0·5–0·25 μ, were found to allow bacteria like *Bacterium coli* and *B. prodigiosum* to pass they must contain pores varying in size from a maximum of the order 1 to 5 μ down to a value well below the stated average. The conclusion was also made that the basic structure of such impregnated membranes was essentially that characterising the gel film

* 'Brit. J. Exp. Path.,' vol. 10, p. 126 (1929).

itself, although no doubt somewhat modified by the distorting influences of the cellulose fibres of the filter paper. This being so, such gel films should exhibit elements of structure comparable in dimensions with the suggested maximum pore values, and, therefore, should prove capable of being detected by microscopic means. The present study was the outcome of these reasonings. Furthermore it deals with an aspect of the problem of ultra-filtration that appears to have been rather overlooked, or too much taken for granted by workers in the past; yet some definite knowledge of the structural nature of membranes is basically necessary for the reliable interpretation of filtration experiments.

Optical Study of Collodion Films.

Numerous preliminary attempts in examining films microscopically by direct transmitted light and also by dark ground illumination, using both stained and unstained preparations, proved unfruitful, except in the case of very coarse structures of precipitated nitrocellulose. No definite result was forthcoming owing to the masking influence of diffraction and general scattering effects. The films were subsequently examined by the aid of the slit ultra-microscope and the results obtained were surprising in their revelation of structural detail. A simple glass cell of the type described by Barnard and Welch* was found very suitable, the fragments of film being covered with a drop of water (see fig. 1). Illumination was effected by a very narrow concentrated beam of light adjusted to almost glancing incidence with respect to

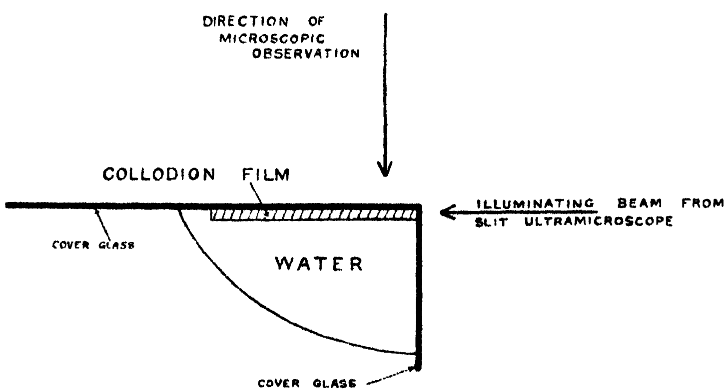

Fig. 1.

the uppermost face of the horizontally disposed film. It was anticipated with the concentrated beam of light used that scattering might again prove an

* 'J. R. Micr. Soc.,' 1925, p. 133.

obstacle. This can easily be the case, and for successful study careful manipulation is necessary and observance of the following all-important points :—

1. A fine steady incident beam of small depth is necessary.
2. Obtain the film in horizontal plane and adjust the incident beam to almost glancing incidence by raising or lowering the microscope stage.

The field obtained shows the path of the beam bright and strongly defined, while on either side the secondary illumination—the result of scattering—falls off in a direction at right angles. Generally, the structure revealed by the main beam properly adjusted to glancing incidence is very clear, but it sometimes happens that here the scattering effects are deterrent, and that better definition obtains in the region illuminated by the secondary light. This is probably due to the fact that the film on either of the main beams is illuminated in a graduated manner by highly polarised scattered light, resulting in the clear revelation of structure. The fallacy so liable to be entertained that high intensity of illumination is prerequisite for the revelation of fine structures is well exposed in this instance. There is obviously an optimum intensity value.

1. *Acetic Acid Collodion Films.*—Acetic acid collodion films prepared upon glass surfaces and also by impregnating filter paper have been subjected to examination by the foregoing optical method. Under comparable conditions (viz., same thickness and gelling procedure) the structure exhibited by these films was found to vary in a definite manner with the concentration of the collodion. Starting with a very dilute solution, say 0·5 per cent. nitrocotton, the fibres are very irregular, the nitrocellulose being more or less in a state of complete precipitation. A typical field obtained with 0·5 per cent. films when examined by the dark ground method is shown in fig. 1, Plate 22. This illustrates the irregular network built up of ultra-microscopic particulate units, which in some parts have clustered together in bunches and in others are linked in strings or fibres. Membranes prepared from such dilute collodions are not suitable for filtration purposes, being much too inconsistent.

As the concentration of the collodion is gradually increased a transition zone occurs, in which the coagulated particles exhibit an increasing tendency to regularity in mutual disposition, linking themselves in chains which anastomose to give a porous sponge-like structure. This latter structure will be referred to as the microgel structure, its elements being microscopic. (Microscopic structures have been observed in other gel systems, viz., coagulated albumen

and gelatin* and silicic acid.) This structure has been observed in films prepared upon glass and also in impregnated membranes, but the close mat of cellulose fibres in the filter paper scatters so much light that observation is rendered very difficult.

However, in order to study films prepared in an analogous manner to that in membrane preparation, Japanese tissue paper was impregnated with collodion and gelled. Here the fibres are less numerous and good stretches of gel can be studied. Plate 22, fig. 2, shows a 1·0 per cent. G.A.C.† film in Japanese tissue paper illustrating well its porous structure. The microgel structure becomes predominant in the region 0·75–1·0 per cent. and then with continued increase in concentration a further structural transition occurs. The films gradually acquire a granular structure, the elements of which are not resolvable—ultragel. The continuous solid phase forming the structural framework expands, its granular consistency becoming increasingly the characteristic structure of the gel, until finally the coarse microgel is completely replaced. This transition suggests that a change takes place in the interaction of the coagulated particles as the nitrocellulose concentration increases, attributable, no doubt, to limitation in freedom of action brought about by the mutual interference of the surface fields of force of adjacent aggregates, desolvation proceeding more uniformly. The variation in structure of the membrane films with concentration of nitrocotton may be conveniently represented diagrammatically as in fig. 2.

As already pointed out, the precise happenings on replacing solvent by non-solvent are dependent upon various factors, such as the relative bulks and distribution of the reactants, and hence the above structural relationships are those manifest under particular conditions, namely, in films of thickness 0·1 to 0·2 mm. In films of 0·01 mm. and less, prepared upon glass surfaces, the microgel structure zone practically disappears. In the case of very thin films, the molecules are undoubtedly orientated in respect to both the collodion/air and the collodion/glass interfaces, surface forces exert an influence practically throughout the entire thickness of the film, and, therefore, on immersion in water the almost instantaneous desolvation permits of no time for mutual interaction and re-arrangement, such as may take place in relatively thick layers of fluid in which the molecules possess considerable latitude of movement.

* Hardy, 'J. Physiol.,' vol. 24, p. 158 (1899), and 'Roy. Soc. Proc.,' B, vol. 66, p. 95 (1900); Bütschli, 'Untersuchungen über Mikroskopische Schaume und das Protoplasma,' Leipzig, 1902; Zsigmondy, 'Z. Anorg. Chem.,' vol. 71, p. 356 (1911); Bachmann, 'Z. Anorg. Chem.,' vol. 73, p. 125 (1912).

† G.A.C. = glacial acetic collodion; E.A.C. = ether-alcohol collodion.

In other words, the conditions in a very thin film are more akin to those obtaining in a very viscous solution, or even to the incipient gel state. In thicker films the microgel zone is extended.

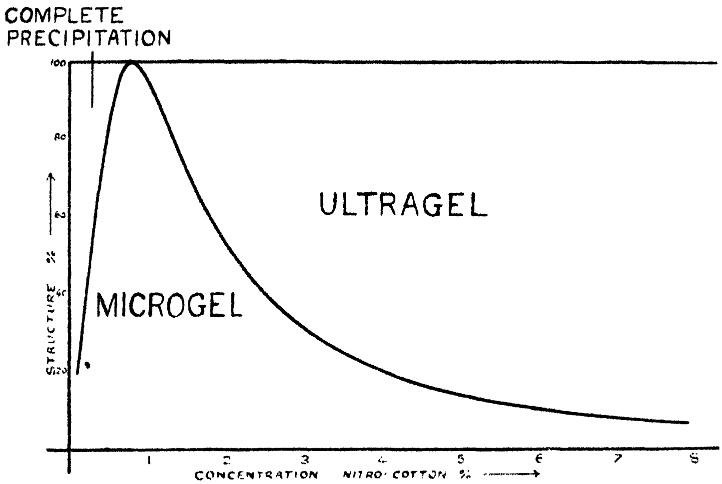

FIG. 2.

Some interesting results upon the relationship between permeability and thickness of gel films have been obtained. Rate of flow of water is not inversely proportional to the thickness of microgel membranes. If the rate of flow for a membrane of thickness x under standard conditions is p, then for a membrane of thickness $2x$, prepared under otherwise the same conditions, the rate will not be $1/2p$, but somewhat greater than this. On the other hand, if two membranes each of thickness x are placed together and the rate of flow through their combined thickness is measured, then proportionability to 1/thickness is found to obtain. The conclusion to be drawn, therefore, is that the relative permeability of microgel films is greater the thicker the membrane.

The velocity of interdiffusion, the dominating factor in the process of this microgel formation, falls off as the thickness increases, so it appears that the slower the desolvating process, the more permeable the film. Further evidence in support of this conclusion is afforded by the fact that, if, instead of washing out the acetic acid in pure water, the process is graduated by using successively mixtures of acetic acid and water in which the proportion of acid is progressively reduced, then the permeability of the resulting film increases. Bechhold and Silbereisen* have recently applied this in a method for obtaining very permeable ultra-filter membranes.

* 'Biochem. Z.,' vol. 199, p. 1 (1928).

Experiments with different Nitrocottons.

The results described were obtained with a pyroxylin of 10·96 per cent. nitrogen content, and the general nature of the structure has been established by the analogous results given by Linter's nitrocotton 10·6 per cent. nitrogen, wood cellulose nitrocotton 10·2 per cent. nitrogen, and lace paper nitrocotton 11·0 per cent. nitrogen. A nitrated viscose 10·6 per cent. nitrogen did not exhibit the microgel structure under the same conditions, the film being exceedingly fragile and microscopically conveying the impression of having been formed by the "flocking" of ultra-microscopic particles, these flocks being held together loosely in the films in uniform disposition. Films from solutions of Schering's celloidin exhibited the microgel structure. Clearly, therefore, the structures described are quite characteristic of permeable acetic acid collodion films, and vary only in coarseness, mechanical strength, etc., properties which are dependent upon the specific nitrocotton used.

2. *Ether/Alcohol Collodion Films.*—Ether/alcohol collodion films examined by the same optical method gave extremely interesting results. The form of experiment adopted was to take a dilute solution, say, 1 to 2 per cent. nitrocellulose in a mixture of equal parts by weight of ether and alcohol, and to pour a known volume over a given area on a horizontal glass plate. The solvent was allowed to evaporate for definite periods, ½ minute, 1 minute, 2 minutes, and so on, the film then being gelled in water.

The results for 1·5 per cent. solutions of Linter's nitrocellulose (10·6 per cent. nitrogen) are contained in Table II—1 c.c. solution was spread over approximately 20 sq. cms. area.

Table II.

Evaporation period.	Collodion, prior to gelling.	Description of films.
Mins.		
0	Fluid	Nitrocellulose precipitated as fibrous mass.
0·5	Fluid	Coarse, irregular opaque film.
1·0	Fluid	Film less opaque and more uniform microgel.
2·0	Fluid	Uniform, but fragile film. Microgel.
3·0	Viscous fluid—incipient gel ?	Uniform, but fragile film—very opalescent microgel → ultragel.
5·0	Set	Uniform film of moderate strength. Ultragel.
10·0	Set	Uniform film and much stronger—almost perfectly transparent. Ultragel.

By progressively increasing the evaporation period, zones of complete precipitation, microgel structure, and then ultragel structure are traversed.

The transition microgel → ultragel is much sharper than in the case of G.A.C. films, owing to incipient gelation. This phenomenon is dependent largely upon the specific solvent. The variations in physical properties of the film as the evaporation period is increased are interesting. A certain degree of opacity characterises films gelled before the onset of spontaneous gelation and this decreases rapidly as the incipient gelling zone is approached, when it merges into the opalescence of the ultragel. This opalescence decreases with longer evaporation and finally the film becomes quite transparent. The tensile strength of the film passed through a minimum value in the microgel zone after which it increases rapidly in the true ultragel zone.

The above observations made with Linter's nitrocotton are typical of the behaviour of ether/alcohol collodion, similar results having been obtained with solutions of the other nitro-celluloses already mentioned, the only variation being one of degree, the microgel zone varying in extent and fineness of structure with the nature of the nitrocotton. Plate 22, fig. 3, shows the microgel structure in the case of the Linter's nitrocotton described, at evaporation period 2·5 minutes. The uniformity of the structure is striking.

Now by the customary methods of preparing ether/alcohol membranes for filtration purposes, incipient gelation is allowed to set in before immersing in water. Hence, the structure characteristic of the resulting film is the ultragel structure, and the microgel which largely characterises the acetic acid collodion membranes is not met with. If coagulation is effected before the incipient gel state, then the microgel structure appears in ether/alcohol films also. Thus the contrast in properties of the two types of membrane given in Table I is clearly explained, and the structural evidence corroborates completely the conclusion drawn from filtration experience.

Ultragel Structure.

A very significant fact already mentioned is that the continuous solid phase in the microgel is granular in appearance and closely resembles, in this respect, the ultragel. It becomes highly desirable, therefore, to have a definite conception regarding the nature of the latter, which possesses the fundamental structure. The optical method used in making the observations so far described does not permit of very great magnification, and a new attack upon the structural nature of the ultragel was made by means of dark-ground microscopy in which far greater magnifications are possible. In these studies the author has had the enthusiastic collaboration of Mr. J. E. Barnard, F.R.S., with his unique technical experience in ultra-violet light microscopy.

Very thin films are necessary for successful dark-ground studies. Two methods were used for preparing these :—

(*a*) A standard drop of collodion placed upon a cover-glass, spread uniformly and then the solvent allowed to evaporate for definite intervals in manner previously described.

(*b*) A standard drop of dilute collodion placed on cover-glass and allowed to drain off quickly while held at 45°. The edge is touched with glass rod to remove last drop and cover-glass immersed in water for working with film on under side. In this way very thin films only a few microns in thickness can be made.

For ordinary dark-ground purposes the films were made upon glass, but for ultra-violet studies quartz cover glasses were used.

Examinations of numerous films on the threshold of the ultragel zone have confirmed the indications of the previous studies in respect to the general structure of the microgel, and have also furnished evidence contributing definitely to our conception of the ultragel structure. The porous nature of the microgel and the granularity of the continuous solid phases are well brought out in Plate 22, fig. 4, which represents a dark-ground field given by an E.A.C. film, using mercury green light as illuminant.

The ultragel is much finer in structure. The most permeable E.A.C. ultragel structures contained elements which were just on the borderline of resolution in the ultra-violet light microscope ($\lambda = 275\ \mu\mu$ cadmium spark used). Observations were best made at edges of the film. Great difficulty was experienced in obtaining sufficiently thin films, and it was felt that by continued experiment upon this technical point alone still more definite and convincing results would follow. However, as already mentioned, the very thin films will most certainly exhibit peculiar structure, since surface forces exert an increasingly powerful influence as the thickness of the film decreases.

The particulate granular nature of the structural units in the coarsest ultragels was definitely established. A certain "grain" or "striated" effect was frequently manifest throughout, as though forces tending to promote an orderly arrangement had been operative during the process of gel formation. Furthermore, the particles in many instances were disposed in chains, in a manner strongly suggesting the existence of an underlying filamentous structure. These points are illustrated in Plate 22, figs. 5 and 6.

As the E.A.C. films became more concentrated by continued evaporation of solvent their transparency increased, and finally they appeared to be optically

void when viewed in the field of the ultra-violet light microscope. This state is reached by a perfectly continuous process, and most probably a progressive change in structural unit occurs, the combination of large aggregate groups gradually becoming less and less the determining factor, being displaced by a still finer structure in which the units are more elementary (viz., molecular instead of polymolecular (micellar) or micellar instead of polymicellar), while an interlacing filamentous structure with fibrils built of condensed molecular or micellar units in parallel orientation may represent the limiting state. This is strongly suggested by the polar nature of the forces operating in the gelling process.

The minute particles as observed in the field of the ultra-microscope behave during the desolvating process as would small magnets, end-on linkages occurring, to give filaments which afterwards form closed structures, the latter representing the more stable distribution of forces. The results obtained by Poole* in his studies upon the physical properties of aqueous gelatin and cellulose acetate in benzyl alcohol gels are very significant. He postulated a network structure composed of minute flexible fibrils with the interstices filled with fluid, and then by mathematical treatment deduced relationships appertaining to the elastic properties of such a system. His experimental data were found to be in good agreement, thus largely justifying the *a priori* assumptions as to the underlying structure in these gels.

General Considerations.

Although the gels considered in the present study are of a particular type, exhibiting many points of difference in physical properties from the more familiar gelatin and agar gels, nevertheless their importance, in view of their extensive use in filtration and diffusion studies, is considerable. The observations recorded here lend support to the views of von Weimarn.† It follows from the precipitation laws of his theory of the colloid state of matter, that the disperse phase may separate in the crystalline state, *i.e.*, complete precipitation, or give rise to gelatinous systems, depending solely upon the concentration of this phase in relation to the solvent properties of the liquid constituent. This is in general accord with the findings of the present study, which provides an excellent example of the manner in which the nature of the resolved system may vary under different conditions. Gelation, in this

* 'Trans. Faraday Soc.,' vol. 21, p. 114 (1925), and vol. 22, p. 82 (1926).
† 'Colloid. Chem.,' vol. 1, p. 27 (1926).

instance, is essentially the product of a coagulating process consequent upon desolvation of the disperse phase of a colloidal system.

The gel consists of two continuous phases, a liquid, water; and solid, or quasi-solid, nitro-cellulose. The solid phase is formed of ultra-microscopic units concerning which the microscopic evidence leads to the tentative conclusion that they are not haphazardly combined, but do tend to regularity in arrangement, determined in ultragel by a still finer underlying filamentous structure and governed by polar forces. The same forces are at work throughout, varying only in degree according to the conditions. The whole mechanism undoubtedly hinges upon the variation and distribution of the free unbalanced surface forces around the dispersed particles, with differing degrees of molecular complexity of the system. The shape of the elementary molecular units is fundamental, and also the spacial relationship of reactive groups.

Ultra-filtration.

The results described are of very definite significance in ultra-filtration. Nitro-cellulose gel membranes prepared by replacement of solvent by water, while the collodion is still in the truly fluid as distinct from the gel state, can exhibit microscopic structure, the elements of which range from 5–10 μ downwards. This structure, as already pointed out, is very prevalent in glacial acetic acid collodion membranes. Consider the permeability curves for such membranes, the form of which is shown in fig. 3. The curve may be regarded in three portions, A, B, and C, as indicated. Section A corresponds to a very permeable region where the change in membrane permeability with concentration is very great. Here the microgel structure is prevalent, and becomes coarser and coarser with dilution of the collodion until it merges into complete precipitation.

Poiseuille's law would scarcely be expected to govern the flow of water through these membranes, and evidence to this effect has been obtained (Elford, *loc. cit.*). Portion B of the curve corresponds to the transition zone, where as the concentration increases the structure becomes more like that of the true gel. Membranes in this region may, although of average pore size (0·5 μ to 0·2 μ), permit bacteria like *B. coli* and *B. prodigiosus* to pass, under suitable conditions—a fact readily explained if the relatively coarse microgel structure still persists, although in progressively diminishing amount. In section C the structure approximates to that of the true gel, *i.e.*, ultragel. The most permeable membranes in this region allow proteins like albumin and globulin to pass, but retain bacteria.

Ether/alcohol collodion membranes, as employed in ultra-filtration and dialysis work, do not exhibit the microgel structure, but are wonderfully

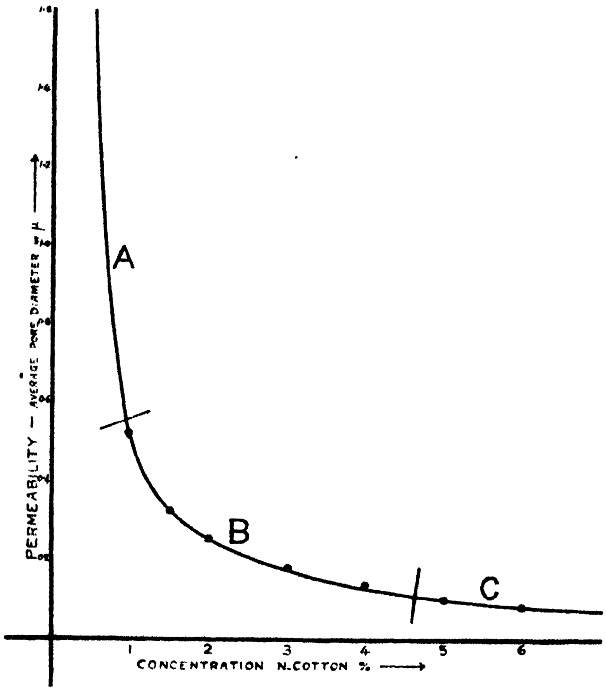

Fig. 3.

uniform and granular in their microscopic appearance. This uniformity is illustrated by the constancy of rate of flow in water measurements, and also by air-pressure tests, when " bells " form in even distribution over the surface and isolated streams of bubbles are not obtained. Membranes of this class can be prepared to allow proteins (albumin and globulin) to pass, and also bacteriophage, but this represents the order of their maximum permeability. This is to be expected with uniform membranes, whose most permeable structures barely come within the limit of microscopic resolution of the ultra-violet light ($\lambda = 275\ \mu\mu$) microscope.

Clearly ultra-filtration, especially with G.A.C. membranes, cannot be the clean-cut process that would be expected of an ideal filter, with all pores of same size. It is this wide range of pore size that makes ultra-filtration results so liable to misinterpretation and renders necessary the strictest adherence to definite technique in all comparative filtration work. Even so differentiation between, and the assignation of size values to, disperse particles by the use of

such ultra-filter membranes is not a simple problem, but on the contrary involves the detailed consideration of many influencing factors.

Summary.

1. Optical studies of permeable collodion films have revealed the existence of two very definite types of structure—(i) microgel structure of microscopic order, and (ii) ultragel structure of ultra-microscopic order. The general nature of these structures has been established for several different nitro-cottons and the conditions determining them investigated.

2. Gelation in this particular instance is a process of phase transition resulting from the coagulating influence of desolvation. The precise nature and stability of the gel is a function of the specific characters of the nitro-cellulose and solvent, and the variation in free surface forces around the dispersed phase particles with differing degrees of molecular complexity of the system.

3. The bearing of these observations upon ultra-filtration has been discussed. A complete explanation of the general behaviour of ultra-filter membranes is afforded, and a sound basis established for the interpretation of filtration results.

In conclusion the author would express his thanks to Mr. J. E. Barnard, F.R.S., for his interest and co-operation in this work; and also to Mr. C. W. Frost, of the R.N. Cordite Factory, Horton Heath, for kindly providing samples of nitrocotton.

EXPLANATION OF PLATE 22.

Fig. 1.—0·5 per cent. G.A.C. Dark ground. × 1200.

Fig. 2.—1 per cent. G.A.C. in Lens paper. Slit ultra-micro. × 150.

Fig. 3.—1·5 per cent. Linter's E.A.C. Evaporation 2·5 minutes. Microgel structure. Slit ultra-micro. × 150.

Fig. 4.—1·5 per cent. Linter's E.A.C. Evaporation 3 minutes. Microgel → ultragel. Dark ground. × 1800.

Fig. 5.—G.A.C. ultragel, from viscous fluid. Shows the particulate nature. Ultra-violet light micro. × 2000.

Fig. 6.—E.A.C. ultragel, after incipient gelation. Shows towards edge of film the "grained" or "striated" structure. Dark ground. × 1800.

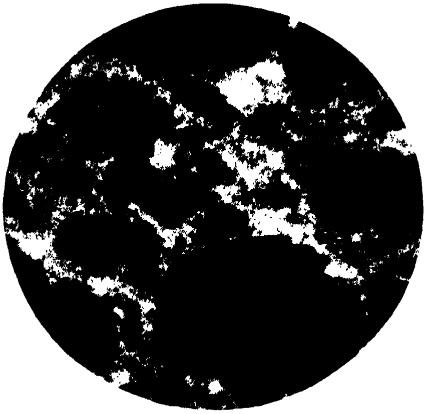

Fig. 1.

Fig. 2.

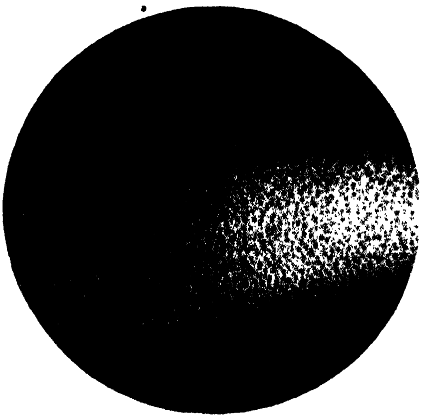

Fig. 3.

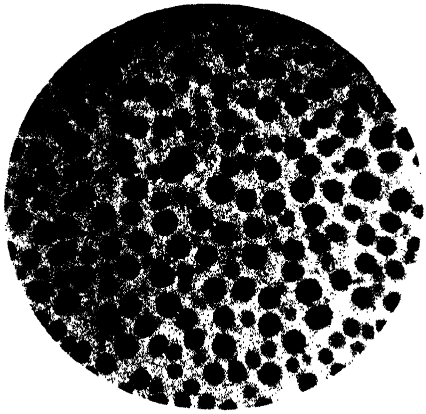

Fig. 4.

Fig. 5.

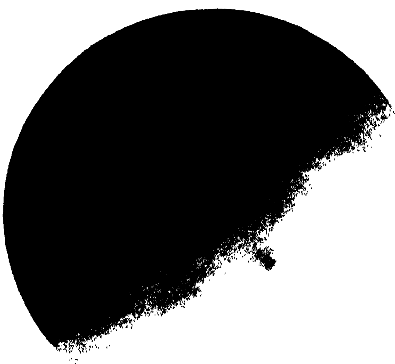

Fig. 6.

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A Theory of Tracheal Respiration in Insects.

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Introduction.

A satisfactory theory of tracheal respiration would not only be of considerable academic interest but, since respiratory poisons are employed for the destruction of many harmful insects, it might prove of great practical value.

Physiological studies on the tracheæ of insects have aimed chiefly at establishing, on the one hand, the mode of ending of these air-containing tubes, and, on the other, the forces which maintain the supply of oxygen to their terminations. As regards the former of these problems, there is no general agreement; for most of those who have studied the subject have worked with different organs from different insects, and almost all have assumed that the farthest point to which they have succeeded in tracing the tubes is in fact their termination. In certain cases, however, there is no doubt that the tracheal capillaries or tracheoles penetrate within the cytoplasm of the tissue cells (see Wigglesworth, 1929).

Again, there has been much divergence of opinion as to whether the terminal portions of the tubes contain liquid or air. Many of the earlier observers (Schultze, 1865; Wielowiejski, 1882; Emery, 1884) considered that the ultimate branches contained liquid, and v. Wistinghausen (1890) believed that this liquid flowed in and out of the cells, so constituting a form of inspiration and expiration. Pantel (1898), however, stated that during life the tracheoles always contain air, and that where fluid had been observed it had entered after death. This view has been accepted by almost all subsequent authors (Keilin, 1924; Remy, 1925); though Koeppen (1921) noted that, under ordinary conditions, the tracheoles in *Dytiscus* contain liquid, and Davies (1927) thought that this might also be the case in *Sminthurus*. It will be shown conclusively in the present paper that they do in fact contain a variable quantity of liquid.

As regards the second problem, the forces which determine the supply of

oxygen to the tracheal endings, Krogh (1920) has shown, in a valuable paper, that the laws of diffusion of gases will explain the supply to the tissues of insects of those quantities of oxygen which they actually consume ; though in certain cases simple diffusion is supplemented in the largest tracheal trunks by mechanical inspiratory and expiratory movements. Krogh, however, dealt only with the tracheæ (the larger tubes with spirally thickened walls) and not with the tracheoles (the ultimate branches which have uniform walls). The latter are so fine that they did not prove amenable to mathematical treatment, and Krogh simply assumed, probably with perfect justice, that in these finest twigs, also, diffusion would account for the gaseous exchanges.

This theory is satisfactory so far as it goes, but it is essentially static. Except where the insect is provided with tracheæ which are mechanically compressible, so that inspiratory and expiratory movements occur—and Krogh himself showed that such tracheæ are by no means universal—it makes no allowance for the exigences of respiratory metabolism, and more particularly it makes no provision for increased requirements locally.

For if it be accepted that the terminal portions of the tracheal system in the tissues contain liquid, then, other things being equal, the oxygenation of the tissues will increase as the extent of this liquid up the tubes becomes less; for the further the liquid extends, the more remote will become the column of air from which the oxygen must diffuse. Now the demand for oxygen will increase with any increase in the activity of the tissues. Therefore, what is required by the insect is that the column of air shall extend more deeply into the tissues when and where their activity is greatest.

It is the purpose of this paper to enunciate a theory, complementary to that of Krogh, which shall satisfy these requirements, and to bring forward some experimental evidence in its support.

Theoretical Considerations.

If it be assumed that the endings of the tracheal tubes are closed (fig. 1, A) and that they are bounded by a membrane which is semi-permeable with respect to lactic acid and similar metabolites, then water will be driven into the tubes by the hydrostatic pressure in the tissues and drawn along them by capillarity, until its progress is arrested by the pressure of gas in the tracheæ and the osmotic pressure of the tissue fluids. Normally, the hydrostatic pressure in the tissues, and the pressure of gas in the tracheæ, will not differ greatly from atmospheric pressure, and the important forces from a quantitative point of view will be osmotic pressure and capillarity.

Of these forces the only one which may be expected to vary with the activity of the tissues is the osmotic pressure. For it is well known that physiological

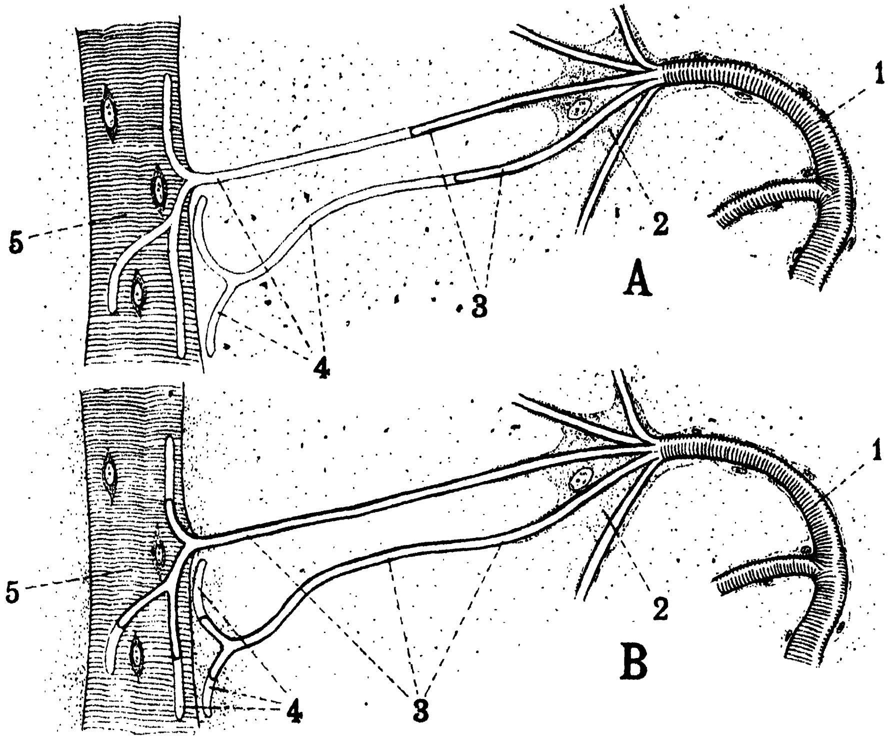

FIG. 1.—Diagrams to illustrate the theory of tracheal respiration. *A*, tracheal ending in resting muscle; *B*, in active muscle. 1, trachea; 2, tracheal cell; 3, parts of tracheoles containing air; 4, parts of tracheoles containing liquid; 5, muscle.

activity is frequently associated with the breakdown of large molecules into small, the most familiar example being the production of lactic acid from glycogen during muscular contraction. Therefore, with increased activity (fig. 1, B), the osmotic pressure will rise, and water will be absorbed from the tracheal tubes. Consequently, the column of air from which the oxygen must diffuse will extend more deeply into the tissues, entering finer and finer tubes until the increasing capillarity of these tubes balances once more the increased osmotic pressure. Moreover this change will take place first in those regions where the demand for oxygen is greatest.

With the process of recovery the normal composition of the tissue fluids will be restored, the osmotic pressure will fall and liquid will rise once more in the tracheal tubes until the position of equilibrium is regained.

Observations and Experiments.

Observations on the Tracheæ and Tracheoles of the Mosquito Larva.

In order to prove this hypothesis it was necessary to observe the tracheal endings in a living insect under varying conditions of activity and oxygen supply. For this purpose an ideal experimental animal was discovered in the larva of the Yellow Fever Mosquito (*Aedes* (*Stegomyia*) *argenteus*). The integument of this insect is but slightly pigmented, so that if it be examined in the living state, under a coverslip supported so as to retain the larva in one position, it is possible, under the one-sixth inch objective of the microscope, to obtain an uninterrupted view of the nerves and muscles within the head capsule, and to follow the tracheal tubes by which they are supplied.

The most convenient trachea to observe is a small branch which arises from the main tracheal trunk to the dorsal aspect of the head soon after this trunk has crossed below the optic nerve, and which then runs outwards towards the eye and the antennal muscle. This branch, which is shown in fig. 2, has been employed for most of the observations recorded below; although these have been confirmed using other tracheæ in the head and elsewhere.

The larger tracheal branches appear as refractile tubes; but where the lumen becomes small they are seen only as slender black threads (fig. 2). It is often possible to see clearly the abrupt termination of the ringed "trachea" proper, which enters the body of a large web-like "tracheal cell" and there breaks up into a number of "tracheoles" with homogeneous walls. The trachea itself almost always contains air, and this extends for a variable distance down the tracheoles. These may be visible as very fine black threads branching and rebranching around the muscles; but usually, after a short course, the air-containing thread ends abruptly and thenceforward the tracheole becomes invisible. Or, if the lumen of the tracheole at this point be sufficiently large, it may be traced, very faintly, a little further towards the tissues although it no longer contains air.

Clearly the terminal portions of the tracheal system contain liquid, and often, particularly in larvæ which have been kept at rest (*i.e.*, in clean water without food) for some time, this liquid may extend almost to the origin of the tracheoles. The level to which it extends, however, is not constant; for if a given tracheole be kept under observation for some time, in a few minutes maybe, or maybe in half an hour, the air will often be seen to extend further towards the tissues. Evidence will be brought forward later to show that this movement is dependent upon the activity of the muscles supplied.

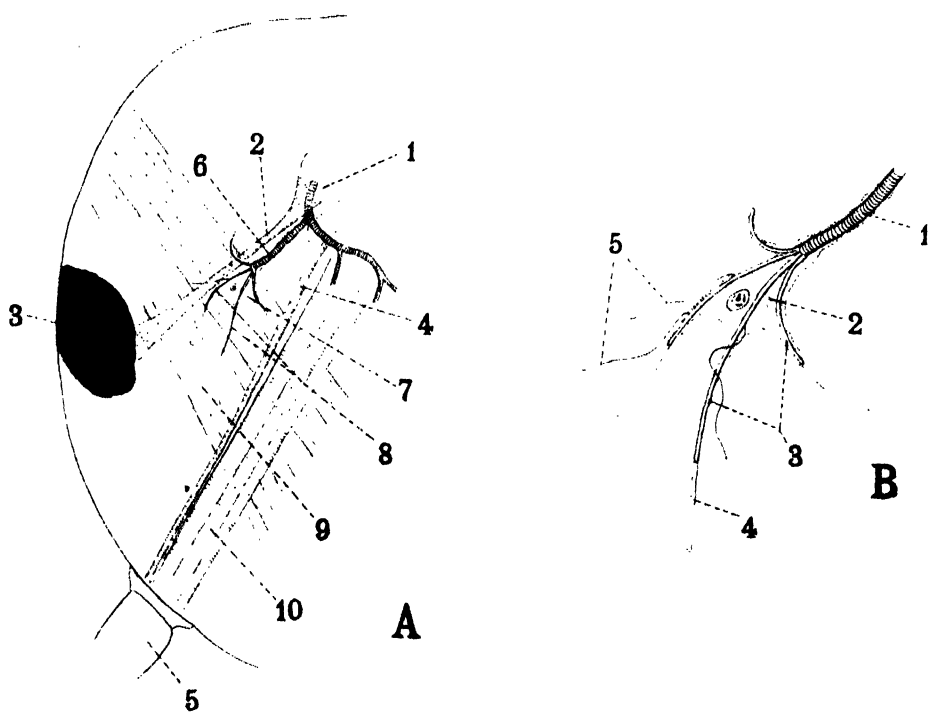

FIG. 2.—A, part of head of third instar larva of *Stegomyia*, viewed from above, as seen in living state, with many anatomical structures omitted. 1, "brain"; 2, optic nerve; 3, eye; 4, antennal nerve; 5, antenna; 6, trachea; 7, tracheal cell; 8, tracheole containing air; 9, tracheole containing liquid (scarcely visible); 10, antennal muscle.
B, detail of tracheal ending. 1, trachea; 2, tracheal cell; 3, main tracheoles containing air; 4, main tracheoles containing liquid; 5, fine tracheoles containing air.

The Effect of Asphyxiation on the Liquid in the Tracheoles.

The mosquito larva has another great advantage for the present purpose in that the tracheal system is open to the exterior at one point only—the respiratory syphon on the eighth abdominal segment. Consequently, the supply of air and the access of other gases may be very readily controlled. For example, if a larva be held beneath a coverslip, and then flooded with water so that its respiratory syphon can no longer reach the surface, it soon becomes violently excited and the muscles within the head twitch vigorously. After a few minutes, the liquid in the tracheoles which supply these muscles begins to be absorbed, and the column of air creeps steadily along the tubes, branching and rebranching, until eventually it enters capillaries so fine that they can no longer be resolved under the one-sixth inch objective of the microscope.

The time required for the movement of the air to begin, and the time required to fill the entire system, varies with the activity of the muscles, and this again varies with the temperature. Fig. 3 represents a typical experiment, in which

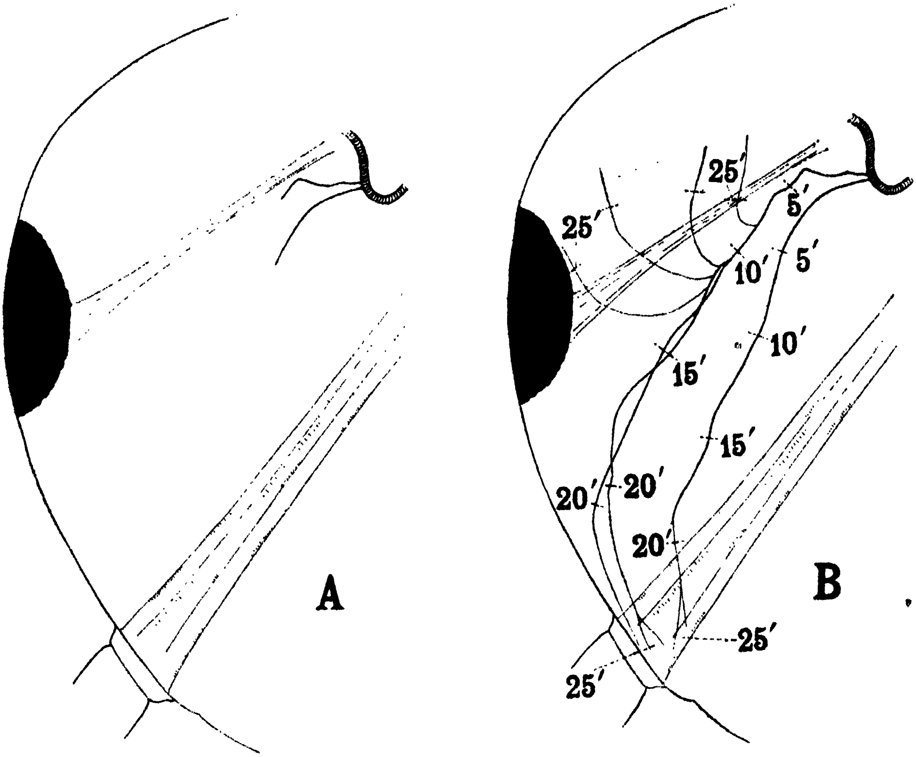

FIG. 3.—Effect of asphyxiation on air in tracheoles of a third instar larva. A, larva at rest; B, during asphyxiation. The figures in B show time in minutes, after onset of asphyxiation, at which air reached points indicated.

A shows the extent of the air at the commencement of the experiment, B at its termination, and along the tracheoles in B are marked the points to which the air extended at different intervals of time. As the tracheole approaches the tissues, it may taper gradually; but more often, after reaching a certain size, it becomes narrowed abruptly to a hair-like capillary which is all but invisible. An attempt has been made to show this change in the lower branches in fig. 3, B. If, at the end of the experiment, the larva be returned to clean oxygenated water, the liquid slowly rises in the tracheoles, and in a few hours has usually regained approximately the level from which it started.

The movement of the column of air towards the muscles in the head during asphyxiation usually commences within 2 or 3 minutes. If the muscles are

inactive, the movement is delayed; and, by the same token, the movement occurs much later towards inactive tissues. Fig. 4 shows the apical portion of one of the anal gills of a larva at the commencement of an experiment and

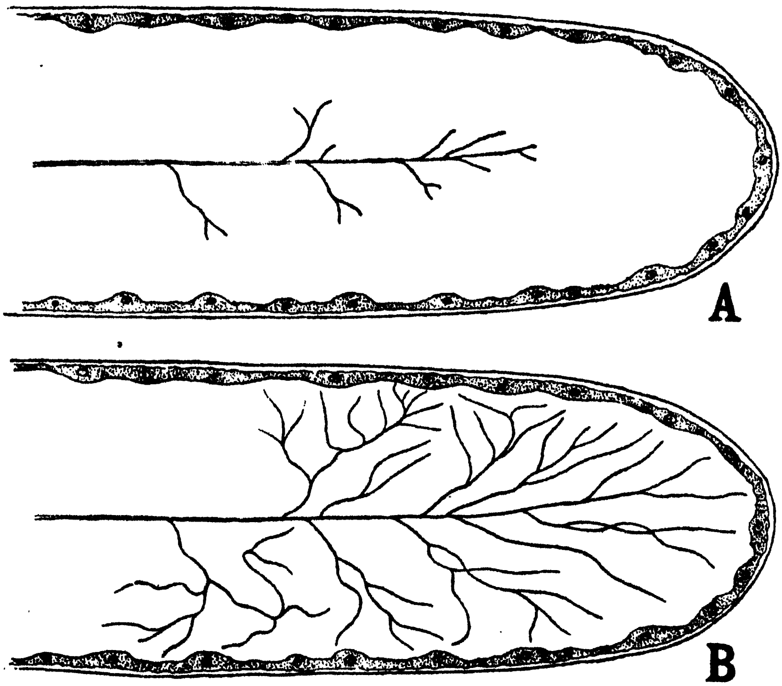

Fig. 4.—Effect of asphyxiation on air in tracheoles of anal gills. A, after asphyxiation for 15 minutes; B, after 30 minutes.

after asphyxiation for half an hour. In this larva, absorption of liquid from the tracheoles of the head began in 2½ minutes, but there was no change in the anal gills for 15 minutes. It is uncertain whether the absorption of liquid from the tracheoles in the anal gills is dependent on the activity of the cells to which they run, or results from muscular contractions elsewhere in the body; but, in view of certain evidence to be brought forward later, the latter notion appears the more probable.

Accumulation of Lactic Acid during Asphyxia.

According to the hypothesis outlined above, the removal of fluid from the tracheoles during asphyxia is to be attributed to the production of metabolites which are osmotically active. Now it has been shown by Davis and Slater (1928) that in the cockroach, during asphyxiation, lactic acid accumulates,

and the insect "goes into debt" for oxygen to an amount approximately equal to the quantity required for the oxidative removal of the lactic acid formed. This suggests that lactic acid is the chief metabolite to accumulate under these conditions.

That lactic acid is produced by the mosquito larva during asphyxiation can be shown readily enough. A number of larvæ from the same culture were kept without food in several changes of clean water for a few days, in order to eliminate fermenting materials from the intestine. A larva was then removed to a waxed tile, dried with blotting paper and cut open in a measured volume (a few cubic millimetres) of bromo-thymol blue. This gave a blue-green colour (pH about 7·0). A similar larva asphyxiated for half an hour gave a greenish yellow colour (pH about 6·4). The experiment was repeated using a very dilute solution of ferric chloride in place of the indicator. A faint lemon yellow tint was given by the asphyxiated larva but not by the control. A far more intense yellow colour could be obtained by using two larvæ and asphyxiating them for an hour.

Effect of Changes in Osmotic Pressure on Liquid in Tracheoles.

It must next be shown that changes in the osmotic pressure of the tissue fluids will affect the level of the liquid in the tracheoles.

The first experiments along these lines were carried out with different concentrations of sodium chloride, the procedure being as follows:—A freshly isolated larva was examined under a coverslip, and a drawing made of the tracheal branches on one side of the head. The larva was then dried with blotting paper, the opposite side of the head was punctured with a fine needle, the solution to be tested was added and the tracheal branches selected for observation were re-examined as quickly as possible. These experiments are open to the objection that asphyxiation may play a part in the results obtained, but, in practice, the effects are produced much sooner than those brought about by simple asphyxiation; they are in fact almost instantaneous

It was found that solutions of sodium chloride of 10 per cent, 5 per cent. and 1 per cent. strengths brought about a rapid extension of air into the tracheoles, and in favourable experiments these might be filled with air as completely as they are during asphyxiation. 0·5 per cent. sodium chloride caused no change unless the muscles were contracting very actively, when the air moved down a little. Distilled water, when applied in the first instance, usually had no effect; but in some cases the liquid in the tracheoles rose somewhat, and the air retreated.

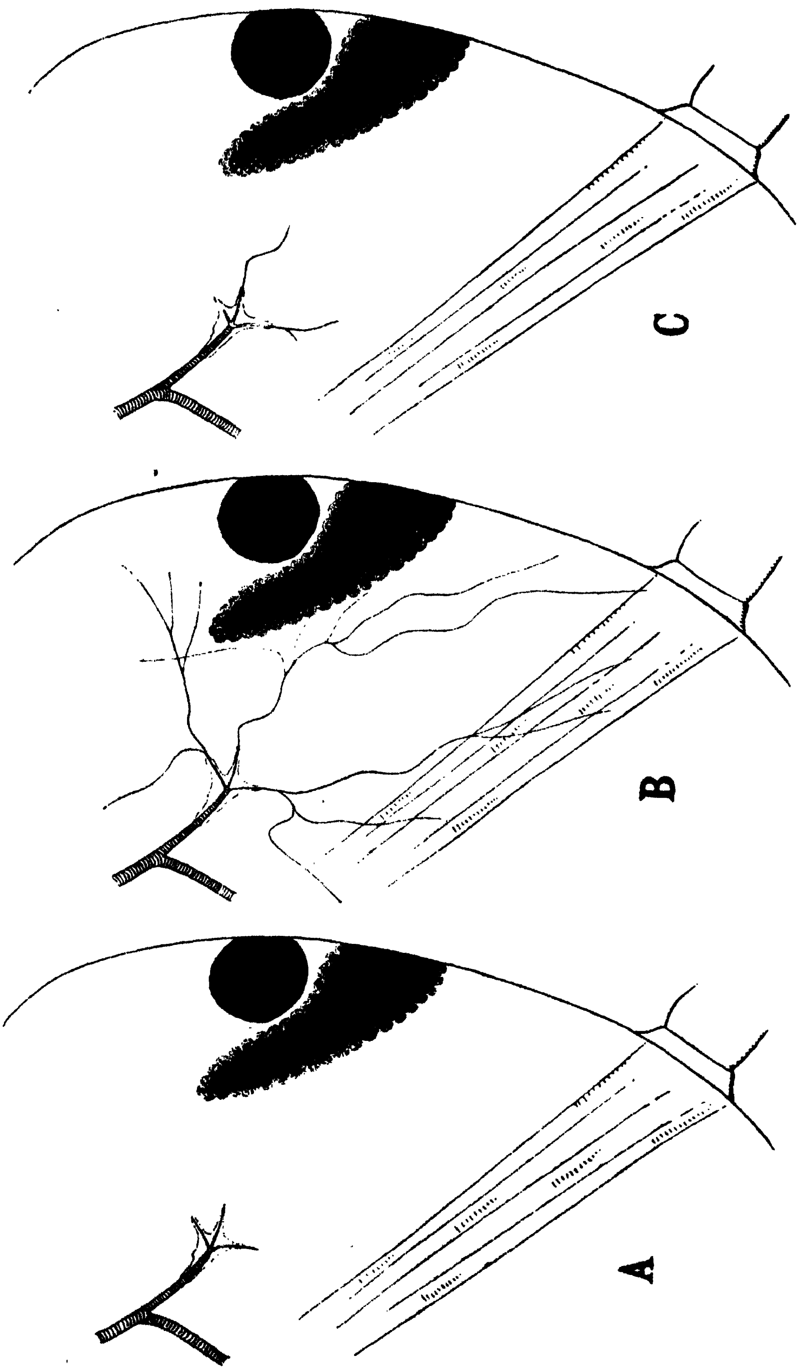

Fig. 5.—Effect of hypertonic solution of sodium chloride on air in tracheoles of a fourth instar larva. A, larva at commencement of experiment; B, immediately after application of 1 per cent. sodium chloride; C, half-an-hour after addition of distilled water.

Fig. 5 shows an experiment of this series in which 1 per cent. sodium chloride was used. At the end of 15 minutes (fig. 5, B) the salt solution was removed and distilled water added. Whereupon the liquid rose very slowly, and at the end of half an hour (fig. 5, C) had risen, in some branches, even higher than at the commencement of the experiment.

Similar experiments have been performed with different strengths of lactic acid and potassium lactate. In the early experiments, when a strong solution of lactic acid was used, a most striking effect was produced. Immediately after application of the solution, the column of air in the tracheoles instantly shot down into the finest capillaries, but within a few seconds the liquid was observed to be rising rapidly, and in 1 minute it had risen higher than at the outset. Clearly the evacuation of the tubes by osmosis was followed by damage to their walls with a consequent increase in permeability.

In the later experiments, when solutions of potassium lactate adjusted to a pH of about 7·0 were employed, it was found that solutions of approximately 7 per cent., 4 per cent. and 2 per cent. strengths caused removal of liquid from the tracheoles and the extension of air into the finest branches, where it remained until the death of the larva. 1 per cent. potassium lactate was without effect. Since the molecular weight of potassium lactate (128) is approximately double that of sodium chloride (58·5), the osmotic pressure of a 2 per cent. solution of the former will be of the same order of magnitude as that of a 1 per cent. solution of the latter. The two series of experiments therefore gave comparable results.

Fig. 6 shows an example in which the tissues were exposed to a 2 per cent. solution of neutral potassium lactate. This was followed, at the end of 15 minutes, by distilled water, which led to a slow filling of the tracheoles with liquid; so that in 1 hour this had returned, in certain of the branches, as high as at the outset of the experiment.

Effect of Hæmolymph from an Asphyxiated Larva on Liquid in Tracheoles of Resting Larva.

It has yet to be shown that the changes in the osmotic pressure of the tissue fluids during asphyxiation are such as will effect the removal of liquid from the tracheoles of a resting larva. This can be shown experimentally without difficulty. Two larvæ of the same age are removed from the same culture. One is asphyxiated in the manner already described, for half an hour. A tracheal branch on one side of the head of the resting larva is selected for observation, and a drawing of it is made. Both larvæ are then dried with

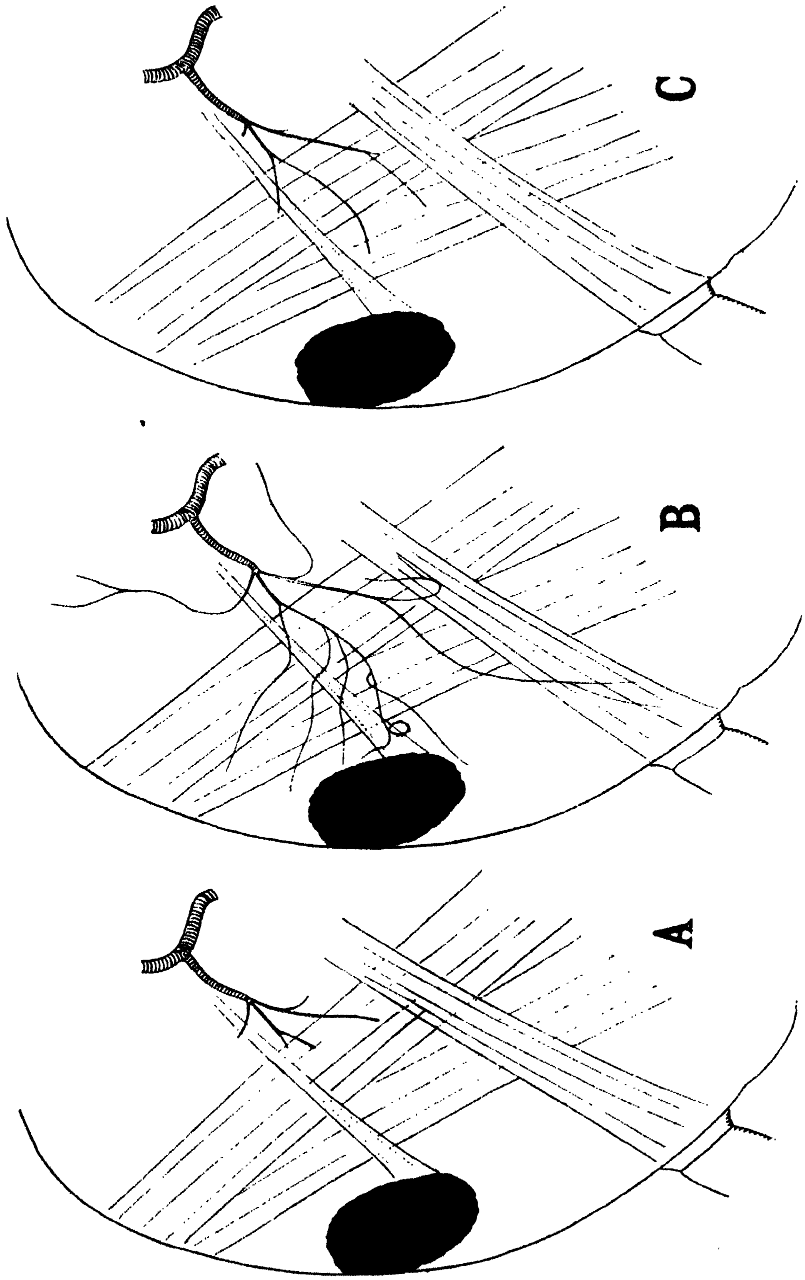

FIG. 6.—Effect of hypertonic solution of potassium lactate on air in tracheoles of a third instar larva. A. larva at commencement of experiment; B, immediately after application of 2 per cent. potassium lactate; C, 1 hour after addition of distilled water.

blotting paper and laid together on a dry slide. The head and thorax of the asphyxiated larva and the head of the resting larva are punctured, and both are covered with a supported coverslip.

As soon as the fluid from the asphyxiated larva has diffused into the head capsule of the resting larva, the air is seen to move rapidly down the tracheoles of the latter and enter the finest capillaries, although it rarely penetrates so far along them as in a larva which is itself asphyxiated. Fig. 7 shows a typical

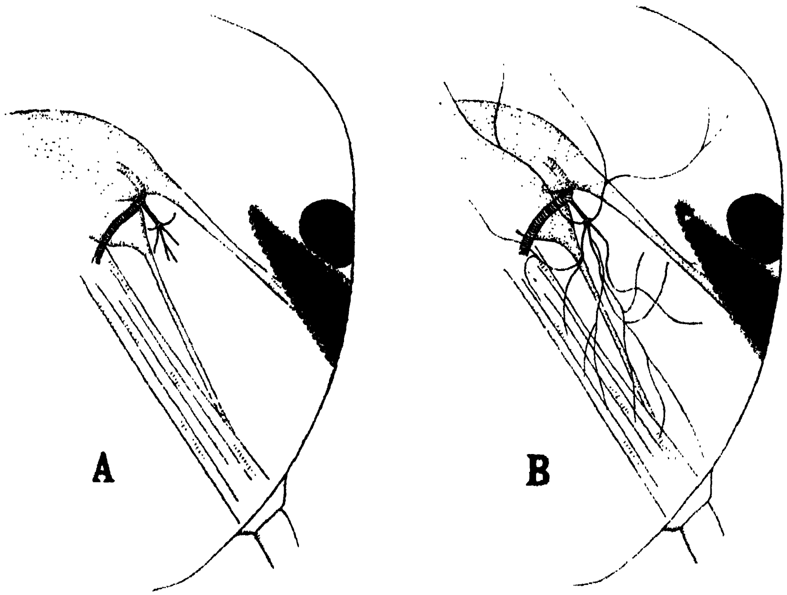

FIG. 7.—Effect of tissue fluids from asphyxiated larva on tracheoles of resting larva. A, resting larva at commencement of experiment; B, after application of fluid from an asphyxiated larva.

experiment of this type. In control experiments, when two resting larvæ are punctured, there is no movement of the air in the tracheoles.

Effect of Exposure to Various Gases.

A series of experiments may now be described, in which the tracheal terminations of mosquito larvæ were kept under observation while the insect was exposed to various gases. For this purpose, the small gas chamber shown in fig. 8 was prepared. A glass platform (A) was secured to a microscope slide (B) with Canada balsam. This platform was surrounded by a ring of " plasticene " (CC′) through which passed glass tubes (D and E). The upper surface of the " plasticene " ring was then pressed flat, to such a level that a coverslip (F) placed upon it would just retain a mosquito

larva (G) upon the platform (A). The union between coverslip and "plasticene" was rendered tight with "vaseline."

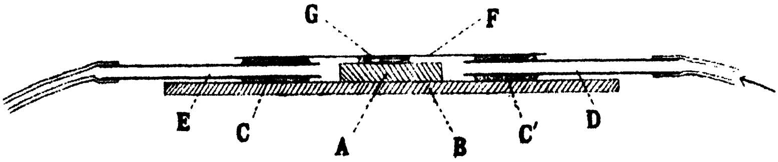

FIG. 8.—Gas chamber for mosquito larva.

The larva was placed in a very small drop of water, so that the respiratory syphon remained open at the surface throughout the experiment; and the gas used, after being bubbled through water so as to saturate it with moisture and prevent evaporation of the drop, was driven through the tube (D) and past the larva.

When a larva is kept in an atmosphere of carbon dioxide under these conditions, the muscles in the head contract rather actively for a few minutes, and the air in the tracheoles can be seen to move down towards the tissues; but within 4 or 5 minutes the larva is completely narcotised, all muscular movements cease, and the extension of the air along the tracheoles becomes arrested before it has nearly reached the finest capillaries. When removed to a vessel of fresh water, the larva soon recovers and, at the end of half an hour or so, the liquid in the tracheoles will be found to have returned towards the original level.

Hydrogen has not the narcotic action of carbon dioxide; consequently the muscles continue to contract for 10 or 15 minutes before the deprivation of oxygen renders the larva inactive. During this time the gas extends slowly down the tracheoles, but although it penetrates further than it does in larvæ exposed to carbon dioxide, it rarely reaches the finest capillaries, as it invariably does during simple asphyxiation. The difference is clearly due to the fact that in the gas chamber experiments the larva has its respiratory syphon open at the surface, so that it remains comparatively placid and does not show the violent contortions of the asphyxiated insect.

These observations afford good evidence that it is the muscular contractions which lead to the absorption of fluid from the tracheoles. Further evidence of this is given when the larva, inactivated by hydrogen, is exposed to oxygen. In less than 30 seconds the larva is restored, the muscles begin to contract actively and the column of air extends further down the tracheoles. With continued exposure to oxygen, the larva becomes quieter, and although the

muscles contract from time to time, the liquid in the tracheoles rises slowly once more towards the original level. An experiment of this type is shown in fig. 9, the time relations and experimental details being given in the legend.

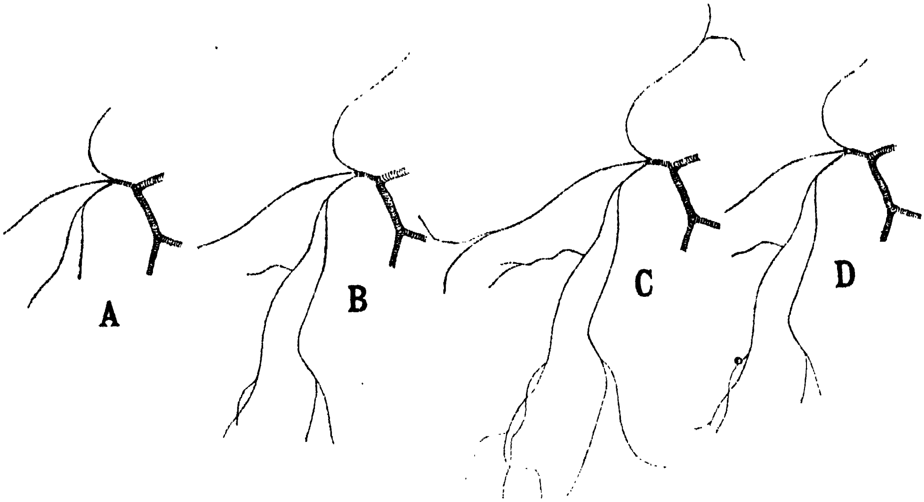

FIG. 9.—Effect of hydrogen and oxygen on the gas in tracheoles of mosquito larva. A, tracheal ending at commencement of experiment; B, after exposure to hydrogen for 20 minutes; C, 3 minutes after admission of oxygen; D, 30 minutes after admission of oxygen.

At first sight this result seemed paradoxical; but it is readily explained by supposing that the central nervous system of the larva is restored by the oxygen before this has attained a sufficient tension in the tissues to prevent the accumulation of metabolites.

Effect of various Poisonous Gases.

In the hope of throwing light upon their mode of action, various gases which are commonly used as insect poisons were administered to mosquito larvæ while the tracheal endings were under observation. The experiments were made in the gas chamber already described, air being drawn, by means of a filter pump, through a wash-bottle in which the gas in question was generated, and then through the gas chamber.

With all the poisons examined, the extent to which the air moves down the tracheoles depends upon the degree of muscular activity which precedes the death of the insect; and soon *after* death (as evidenced by the cessation of all movement) the liquid begins to rise in the tracheoles, and eventually reaches a point above that occupied in the living larva. In other words, none of these

poisons appears to have any specific action upon the tracheal system, and the properties of the membrane bounding the tracheal tubes are not affected until the insect dies. The permeability then increases, and the terminal portions are soon filled with liquid.

To record very briefly the results obtained : *Chloroform* acted comparatively slowly ; the muscles contracted actively for about 2 minutes, the liquid began to rise again in the tracheoles in about 10 minutes, and was almost back to the original level in half an hour. *Carbon disulphide* acted similarly. *Hydrogen cyanide* was more rapid in action. There was little muscular movement, and in half an hour the liquid had risen higher than at the outset. In *ammonia* there was scarcely any muscular contraction ; the larvæ were dead in about 10 minutes and the fluid in the tracheoles rose higher than at the outset almost at once. *Sulphur dioxide* and *chlorine* killed the larvæ in 2 or 3 minutes, and the liquid then rose, usually very rapidly, above the original level.

The Effect of Oil in the Tracheal System.

Since " oiling " is the classical method of killing mosquito larvæ, and since this acts by way of the tracheal system, it was of interest to observe the tracheal endings in larvæ exposed to oil.

The effects can be illustrated best by describing a single experiment. At 4.30 p.m. a larva was held beneath a coverslip, in a drop of water, with its syphon open at the surface, and a drop of kerosene run in so as to come in contact with the syphon. The kerosene entered the tip of the syphon almost at once ; and at 4.33 p.m. it was seen to enter the left tracheal trunk of the body and extend steadily down it, until this was completely filled with oil as far as the thorax. The movement occupied only a few seconds, and no bubbles of gas escaped from the larva in the process. At 4.35 p.m. the right tracheal trunk filled in the same manner. By 4.45 p.m. the oil had entered the tracheoles of the head and only the finest capillaries still contained gas. The transverse trachea, which connects the two main trunks in the thorax, was seen to fill with oil from the two ends simultaneously. By 4.47 p.m. all gas had vanished from the tracheal system, the finest capillaries being filled with oil. At 5.0 p.m. the muscles were still giving occasional twitches.

The complete disappearance of gas from the tracheal system is remarkable to witness, but the explanation is probably very simple. The oil has a strong affinity for the lining membrane of the tracheæ, as may be judged from the highly concave meniscus at the free surface of the liquid as it moves along the tubes. The surface tension of the oil will therefore exert pressure on the gas

in the tracheal system, and this gas will be driven into solution in the tissue fluids, eventually diffusing to the exterior. The solubility of the gas will be increased also by the removal of oxygen by the tissues and the consequent increase in the partial pressure of nitrogen. The experiments are interesting chiefly as showing what striking effects may be produced by simple physical forces when they act in a system of this magnitude. They serve to show also that the tracheal walls, quite apart from the tracheoles, are freely permeable to oxygen and nitrogen; for no tracheoles were given off by the tracheal branches which were seen to fill from both ends simultaneously.

Similar observations were made by Freeborn and Atsatt (1918) and Hacker (1925), but none of these authors makes it clear that air may be expelled completely from the tracheal system.

Observations on other Insects.

With the object of showing that the mechanism described is of general occurrence, a few observations have been made on other insects. In the first place, the flight muscles of the blow-fly (*Calliphora*) were examined immediately after isolation from a resting insect and from an insect asphyxiated for half an hour; but the supply of air-containing tubes is so exceedingly abundant even in the resting condition that it was not possible to detect any difference with certainty.

The mid-intestines from several larvæ of the dragon-fly (*Aeschna*) which had not been fed for nearly 3 months, were then examined, being mounted immediately after removal, moistened only with the tissue fluids of the insect itself. The air-containing tracheoles derived from each tracheal trunk ended abruptly, just like those of the mosquito larva; and on the addition of hypertonic sodium chloride or potassium lactate the air extended rapidly along them. Fig. 10 represents a small area of the gut surface before and after the addition of 3 per cent. potassium lactate.

In the fasting larva, the tracheal supply appeared comparatively scanty; and between the terminations of adjacent trunks were considerable areas in which no tracheoles were visible. This appearance was in marked contrast to that seen in several larvæ dissected 2 hours after a copious meal of blow-flies. In these the entire surface of the gut was covered with tangled skein of air-containing tracheoles; and here and there these seemed to dip down and form little basket-like clusters around or within the epithelial cells.

Although these changes are not so convincing as those which can actually be witnessed in the mosquito larva, it seems probable that in these insects,

also, activity of the tissues is followed by an extension of air towards the tracheal endings.

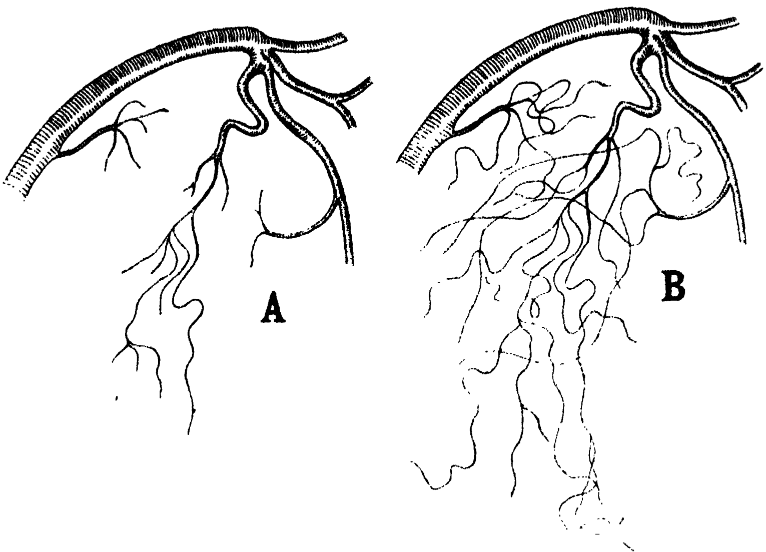

FIG. 10.—Effect of hypertonic solution of potassium lactate on tracheal endings in mid-gut of *Aeschna* larva. A, tracheal endings immediately after dissection; B, after application of 3 per cent. potassium lactate.

DISCUSSION.

The experiments described have shown conclusively that the changes foreshadowed on theoretical grounds not only do take place, but are of sufficient magnitude to influence the oxygenation of the tissues very materially. This may be seen at a glance by comparing A and B in fig. 2; and the difference remains material despite the exaggeration in these figures, which results from the branches being depicted all in one plane and considerably thicker than they would appear at that magnification.

The conclusion that the movement of liquid in the tubes is caused by a direct osmotic effect of lactic acid (or some equivalent metabolite) is perhaps not so sure. A membrane impermeable to a lactate is not a structure with which biochemists are familiar; so that any change in osmotic pressure due to this substance would be expected to be temporary only.* The possibility

* It should be noted (fig. 6) that even in distilled water the return of liquid up the tracheoles is relatively slow; so that a temporary change in osmotic pressure may lead to a prolonged change in the level of the liquid. Further, it has recently been found by Prof. A. V. Hill (unpublished work, to which I am kindly allowed to refer) that in frog

remains that during muscular contraction, the lactic acid set free and the carbon dioxide produced may affect the osmotic pressure in virtue of their acidity; either by altering the dispersion of the proteins, or indirectly by affecting the imbibition of water. But since the removal of liquid from the tracheoles can be induced by hypertonic solutions of lactate even when these solutions are *neutral*, it has not been considered necessary to discuss these more complicated mechanisms. Moreover, when larvæ were exposed to carbon dioxide, the absorption of liquid was arrested as soon as the insect became narcotised and muscular contractions ceased.

It has been assumed throughout that the surface tension of the body fluids remains constant; but in view of the work of Hartridge and Peters (1922) showing the great changes in tension at an oil-water interface which follow slight changes in hydrogen ion concentration, the possibility remains that effects due to changes in surface tension may not be negligible.

In certain respects the observations recorded create more problems than they solve. In the theoretical treatment it was postulated, for the sake of simplicity, that at the outset the tracheal tube, bounded by a semi-permeable membrane, contains air; and that in virtue of simple physical forces it comes to contain a liquid which must be a solution in water of such constituents of the tissue fluids as can pass through the membrane. By hypothesis (and by experiment) these constituents cannot have molecules larger than that of lactic acid.

Now it has long been known that on emergence from the egg, and, in many cases, after each ecdysis, the tracheal system is filled with liquid. Keilin (1924) and Davies (1927) showed that, after hatching, this liquid is absorbed into the tissues, and thenceforward the system contains air. What then is the nature of the tracheal liquid in the newly hatched insect? If it contains proteins and other substances to which the walls of the tracheoles are normally impermeable (*i.e.*, if it has approximately the same composition as the general tissue fluids) then the permeability of the walls must change later, and the fluid must be absorbed by something other than osmosis. Keilin attributes the absorption to "protoplasmic imbibition." But imbibition can affect only the water in the immediate vicinity of the system concerned, and the removal of water from within the tracheal tubes must still take place by osmosis

muscle, stimulated to complete exhaustion, there is a rise of osmotic pressure which may be equivalent to 0·4 per cent. sodium chloride. This is several times greater than would correspond merely to the lactic acid produced; the nature of the substance concerned is unknown.

—the osmotic pressure outside the tube being increased as the result of imbibition. But unless the membrane is semi-permeable with respect to some constituent in the tissue fluids, osmosis cannot occur; and if the tracheal liquid has approximately the same composition as the tissue fluids, the removal of water must soon be arrested by the rise in osmotic pressure within the tube. We are, therefore, driven to the conclusion that absorption of liquid from the tracheal system can take place only if the tracheal membrane is semi-permeable and the tracheal liquid a solution of substances which will pass through it.

If this be so, how does the tracheal system come to contain such a liquid? In the course of development, the tracheæ arise from a series of invaginations of the ectoderm, and they are lined by a cuticular layer which is continuous with the outer cuticle of the insect. The simplest explanation of the phenomena under discussion is that the tracheal liquid is an ultra-filtrate from the tissue fluids, driven through the walls of the tubes by the hydrostatic pressure within the developing egg. On emergence from the egg, the hydrostatic pressure being reduced, the tracheal liquid will be absorbed by osmosis. It would seem unnecessary, therefore, to invoke imbibition, although of course, this may occur.

It is interesting to note also that the activity of the newly hatched insect, by liberating metabolites and so increasing the osmotic pressure, will aid the absorption of liquid and thus accelerate the commencement of tracheal respiration, just as the accumulation of carbon dioxide initiates the respiration of the newborn mammal.

These considerations, however, are at the moment mainly hypothetical, and the chief interest of the present work lies in the fact that it gives to tracheal respiration a dynamic quality which renders it far more adaptable to the changing needs of the organism as a whole, and, more especially, provides for the adjustment of local respiration to meet the demands of the individual organs; just as variations in the capillary bed subserve the same purpose in the internal respiration of vertebrates.

Summary.

A theory of tracheal respiration is put forward, which will provide for the increased demands for oxygen which arise locally in active tissues.

If it be assumed that the terminal portions of the tracheal tubes are bounded by a semi-permeable membrane, then liquid will be drawn up the tubes from the tissues by capillarity until its progress is arrested by the osmotic pressure

of the tissue fluids. During activity lactic acid, and, probably, other substances, will be produced, the osmotic pressure will rise, liquid will be absorbed and air will extend down the tubes towards the active tissues.

This theory is supported chiefly by experiments on the larva of the mosquito. It has been found (i) that in the resting condition the terminal portions of the tracheoles are filled with liquid ; (ii) that during asphyxiation this liquid is absorbed, and the column of air extends rapidly towards the actively contracting muscles—more slowly and much later towards inactive tissues (for example, the anal gills) ; (iii) that on readmission of air the liquid slowly rises to its original level ; (iv) that during asphyxiation an excess of lactic acid is present in the tissue fluids ; (v) that hypertonic solutions of sodium chloride and potassium lactate introduced into the living larva cause extension of air down the tracheoles ; (vi) that the tissue fluids from an asphyxiated larva have a similar effect ; (vii) that hypotonic fluids (distilled water) are without effect, or cause a slight rise of the liquid in the tracheoles.

Some observations are recorded on the effects of certain poisonous gases and the effect of oil on the tracheal system.

My thanks are due to Dr. P. A. Buxton and Prof. R. A. Peters, who kindly read and criticised the manuscript of this paper.

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Appendix.

Quantitative Relation between Osmotic Pressure and Capillarity in the Mosquito Larva.

I am indebted to Prof. A. V. Hill for suggesting that it is desirable to show by calculation that the various forces likely to exist in the mosquito larva are, in fact, capable of bringing about the changes which have been ascribed to them in the foregoing paper. For this purpose we may disregard the hydrostatic pressure in the tissues of the larva, which must be relatively very small, and the pressure of gas in the tracheæ, which, in this insect, will be approximately equal to atmospheric pressure, and consider only the capillarity of the tracheal tubes and the osmotic pressure of the tissue fluids.

The data upon which such a calculation may be based are meagre and uncertain. The diameter of the tracheoles at the upper limit of fluid in the resting larva is difficult to estimate, but in a number of measurements it appeared to vary between about $0 \cdot 5\ \mu$ and $0 \cdot 3\ \mu$; while Davies (1927) gives a value of $0 \cdot 25\ \mu$ in the case of *Sminthurus*. No values are known for the osmotic pressure in the tissue fluids of the mosquito larva, but Bishop, Briggs and Ronzoni (1925), in the larva of the honey-bee, obtained a value for $\Delta = 0 \cdot 86°$.

Now the capillary force in a tube of radius r cm. is given by the expression: $2\pi r\sigma \cos \alpha$, where σ is the surface tension of the liquid in dynes per centimetre and α is the angle of contact between the fluid and the wall of the tube. If it be assumed that the fluid has the surface tension of water (75) and that it wets the wall of the capillary "perfectly," then $\alpha = 0°$ and the above expression becomes $2\pi r \times 75 \times 1$.

If we regard this force as exerted on an area equal to the cross-section of the

capillary, it will be equivalent to a pressure of $\frac{2\pi r \times 75}{\pi r^2} = \frac{150}{r}$ dynes per square centimetre. And if we take $0 \cdot 3\ \mu$ as the average diameter of the capillary at the upper limit of the fluid, this pressure $= \frac{150}{0 \cdot 15 \times 10^{-4}} = 10^7$ dynes per square centimetre. Now the atmospheric pressure is about 10^6 dynes per square centimetre; so this figure approximates to 10 atmospheres.

Turning to the osmotic pressure; a value for Δ of $0 \cdot 86$ would be given by a $1 \cdot 32$ per cent. solution of sodium chloride, which would have an osmotic pressure of $\frac{1 \cdot 32 \times 224}{\frac{1}{2} \times 58 \cdot 5} = 10 \cdot 1$ atmospheres.

In the condition of rest, therefore, the forces of capillarity and osmotic pressure will balance each other. There is little to indicate how great may be the increase in osmotic pressure following muscular activity. Recently, however, Hill (see footnote, p. 245) working with frog muscle, has found an increase in osmotic pressure in muscles stimulated to complete exhaustion, which may be equivalent to as much as $0 \cdot 4$ per cent. of sodium chloride. A change of this order would increase the osmotic pressure by 3 atmospheres, making a total of 13 atmospheres, which (employing the expression given above) would draw the fluid into capillaries with a diameter of about $0 \cdot 2\ \mu$.

It is to be noted that in these calculations the value of Δ is borrowed from an unrelated insect. Also it is assumed that the angle of contact of the liquid with the wall of the tube approximates to zero; that the size of the tube where it is filled with liquid is the same as where it contains air and is exposed to great surface-tension forces; and that the wall of the tube is completely impermeable to all the substances dissolved in the tissue fluids. These are serious assumptions, and too much weight must not be attached to the results; but the calculation may be of some value as showing that the theory put forward is not unreasonable on physical grounds.

The Time Course of the Heat Effects in Rapid Chemical Changes. Part I.—*Apparatus and Methods. Part* II.—*Some Reactions of Acids, Bases, Amino-Acids and Proteins.*

By F. J. W. Roughton, Ph.D., Fellow of Trinity College, Cambridge.

(Communicated by H. Hartridge, F.R.S.—Received November 7, 1929.)

(Abstract.)

Summary of Part I.

(1) The method of Hartridge and Roughton for following the velocity of rapid chemical reactions has been extended to the measurement of the amount of heat liberated in rapid reactions within periods of 0·01 second or less from the commencement of such reactions. For this purpose it has been necessary to carry out an extensive test of the physical sources of error involved in the measurement of temperature of rapidly moving fluids by means of thermo-junctions. As the result of numerous controls and computations it is concluded that with the apparatus described in the text it is possible to measure the absolute temperature of the fluid travelling down the observation tube of the Hartridge-Roughton apparatus to an accuracy of 0·001° C. This claim is confirmed by experiments on the heat evolved in numerous rapid reactions, the values obtained agreeing very closely with the standard accepted values of the heat of such reactions.

(2) Other tests have shown that the method is equally valid for determining the temperature gradients in reacting fluid as it travels down the observation tube, when the reaction under study is slow enough to "occupy" more than the first 2 to 3 cms. of the observation tube. The temperature difference between two points in the observation tube in such circumstances is measurable to an accuracy of about 0·0002° C.

(3) For many purposes a higher accuracy (viz., of 0·0001° C.) is required in the measurement both of the absolute temperature and of the temperature gradient in the observation tube. Modifications of the method are at present being carried out with this end in view, and an effort is also being made to reduce at least 50-fold the volume of reagents required for each experiment, the present requirement being very large, viz., about 4 litres.

Summary of Part II.

By means of methods and apparatus described elsewhere the time relationships of the heat evolved in a number of rapid chemical reactions have been investigated. It was found that :—

(1) The total heat of neutralisation of most of the usual acids and bases (strong and weak) is liberated within a period of less than 0·01 second.
(2) The reactions of carbonic acid with alkali, and of bicarbonate with acid, however, involve heat changes which under appropriate conditions are spread out over periods of considerably more than 0·01 second.

The buffering reactions of glycine and the blood proteins were also studied in a preliminary manner, on account of the physiological interest of these processes. In all cases it was found that the heat changes of the reaction were completed (or almost completed) within 0·014 second, as shown by the agreement between the temperature changes observed to have occurred within 0·014 second and the total temperature changes calculated from the heat of the reaction, as measured by other observers. The physiological and physico-chemical implication of these results is discussed.

A single experiment on the reaction between carbon monoxide and hæmoglobin showed that the heat accompanying this reaction was spread out over a period of 0·05 second.

Further applications of the method are foreshadowed, if and when success has been obtained in extending the accuracy of the temperature measurements from its present limit of 0·001° C. to a further place of decimals, viz., 0·0001° C.

[*The full papers are printed in* " Proceedings," *Series A, Vol.* 126, *pp.* 439-486, *February*, 1930.]

A Thermal Method of Measuring the Vapour Pressure of an Aqueous Solution.

By A. V. Hill, F.R.S.

(Received January 16, 1930.)

(Abstract.)

A thermoelectric method is described by which the difference of vapour pressure between two solutions, or between a solution and the pure solvent, can be measured. The principle involved is simply that of a differential wet-bulb thermometer of high sensitivity. A reading is obtained in 30 to 45 minutes and the average error of a single observation is of the order of 1½ per cent. of the difference read.

[*The full paper is printed in* " Proceedings," *Series A, Vol.* 127, *pp.* 9-19, *April*, 1930.]

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Physiological and Anatomical Evidence for the Existence of Nerve Tracts Connecting the Hypothalamus with Spinal Sympathetic Centres.

By J. Beattie, G. R. Brow, and C. N. H. Long (University Clinic, Royal Victoria Hospital and McGill University, and Department of Anatomy, McGill University, Montreal).

(Communicated by Prof. J. P. Hill, F.R.S.—Received September 2, 1929.)

[Plates 23, 24.]

Introduction.

Levy* (1) in a series of papers has shown that in animals (chiefly cats) under chloroform anæsthesia the heart is particularly liable to show ventricular extrasystoles. These irregularities are often followed by fibrillation. He found that if the irregularity was not present it could be elicited easily by any form of sympathetic stimulation, either direct, reflexly, or through drugs, such as nicotine or adrenaline. Furthermore, removal of the sympathetic nerve supply to the heart along with removal of the adrenal glands rendered the heart immune to this phenomenon. After this procedure reflex stimulation of the sympathetic nervous system was ineffective, but injection of adrenaline readily reproduced the original series of events.

The investigations reported arose out of the accidental finding that decerebration of an animal (cat) at the Sherrington level had a remarkable effect on these cardiac irregularities under chloroform. After decerebration it was as a rule impossible to elicit them with chloroform so long as the adrenal medulla was inactive. Furthermore, if the decerebration was done under chloroform while irregularities were present, they at once disappeared.

The first part of this paper deals with acute experiments, which were designed to locate as accurately as possible the particular area of the brain, removal of which was responsible for the effects described above. This was roughly located in the hypothalamic area, within certain fairly well-defined limits.

In the second part of this paper chronic experiments are recorded, by which

* Although it is not generally recognised, Levy's experiments give a striking confirmation of Cannon's views on the conditions of activity of the adrenal medulla.

we have attempted to trace the degenerations which followed experimental lesions of the hypothalamus. The lesions which were produced in the chronic experiments were identical with those which abolished the irregularities appearing in the heart under chloroform anæsthesia, with the exception that the lesions were unilateral and not bilateral, for reasons which will be explained later.

I. *Acute Experiments.*

Experimental Methods.—All the observations were recorded on cats. They were first lightly anæsthetised with di-allyl-barbituric acid. A tracheal cannula was inserted, to which was attached a pair of respiratory valves, the inspiratory side of which was attached to a large Douglas bag containing 95 per cent. oxygen and 5 per cent. carbon dioxide. The necessary operative procedures were then completed.

When we wished to induce cardiac irregularities the inspiratory valve was switched over to another bag containing the same gas mixture, but to which sufficient chloroform had been added to produce a 2 per cent. concentration of this vapour. In some of our experiments no di-allyl-barbituric acid was injected, but 2 per cent. chloroform vapour used throughout the experiment.

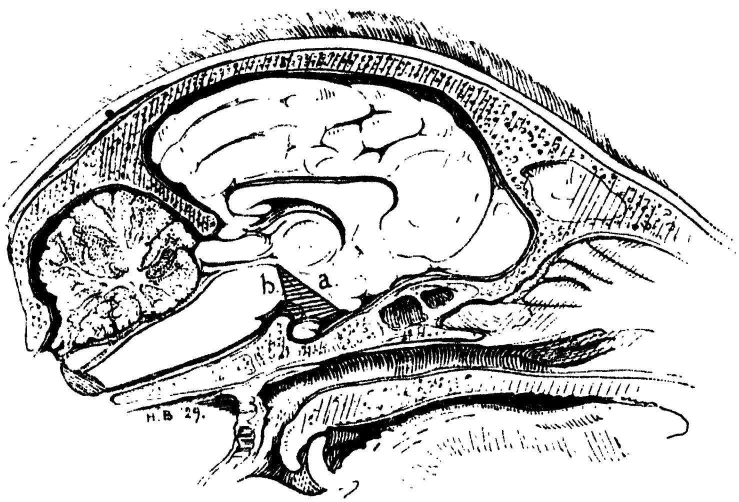
In all our experiments blood pressure and electrocardiographic tracings were taken. The latter tracings were taken by stitching brass electrodes under the skin of the right fore-leg and left hind-leg, so that the curves were comparable to the standard lead 2.

The Effects of Section of the Brain Stem at Different Levels on the Irregularities Produced by Chloroform Anæsthesia.—We first repeated Levy's observations that removal of the stellate ganglia and adrenal glands rendered the heart refractory to irregularities induced by chloroform anæsthesia. We also showed that removal of the stellate ganglia, or section of their connections with the spinal nerves, or section of the anterior roots of the second, third and fourth thoracic nerves in the absence of the adrenal glands, abolished the irregularities when such were present.

As previously stated, we had observed that decerebration of the animal prevents the appearance of the extrasystolic arrhythmia, provided there is no disturbance caused by the injection of drugs or by abnormal sensory stimuli. Further, decerebration leads to the abolition of the extrasystoles when these are present.

We performed a series of experiments to determine the exact level at which the effective section lay. We found that the removal of the cerebral cortex, provided that the thalamus was left intact, had no effect on the irregularities.

When the brain was transected by a cut passing from the anterior edge of the superior colliculus behind, to the posterior edge of the optic chiasma in front, no abolition of the extrasystoles occurred, nor had this section any effect on the induction of the arrhythmia by chloroform after the operation had been completed. When another cut was made from the same posterior and dorsal limit to the level of the mammillary bodies ventrally, the extrasystolic arrhythmia disappeared and could not be made to appear again by the re-application of chloroform. Sections between these two levels did not abolish the arrhythmia permanently (fig. 1).



TEXT-FIG. 1.—A median sagittal section through the head of a cat, to show the relation of the posterior region of the hypothalamus to the base of the brain. The triangular shaded area encloses this region. The anterior line (*a*) is the most posterior section that can be made without interfering with the maintenance of extrasystoles under chloroform. The posterior line (*b*) indicates the approximate level of an effective section to abolish these cardiac irregularities.

Histological examination of the brain after a section between the superior colliculus and the optic chiasma demonstrated that the ventral nuclei of the thalamus were intact, and also the greater part of the ventral portion of the lateral wall of the third ventricle. Furthermore, the globus pallidus had been removed.

To obtain a more accurate localisation of the extent of the effective area, different operative procedures had to be used. The first method was the trans-oral approach to the floor of the third ventricle which has been described in detail by McLean (2). By exposing the whole of the ventral aspect of the

pituitary gland, it was possible either by removing the gland or even without this to produce stab wounds into the brain tissue without disturbing to any great extent the vascular structures in this region. Care had to be taken to prevent damage to the cavernous sinus and the smaller sinuses which surround the diaphragm of the pituitary fossa. Bleeding from these structures was stopped by the application of muscle compresses, but in many experiments medullary hæmorrhage supervened owing to the blood tracking downwards after the external hæmorrhage had been controlled. All experiments which were complicated by this factor were discarded and only those retained in which the presence of medullary hæmorrhage was proved to be absent by autopsy.

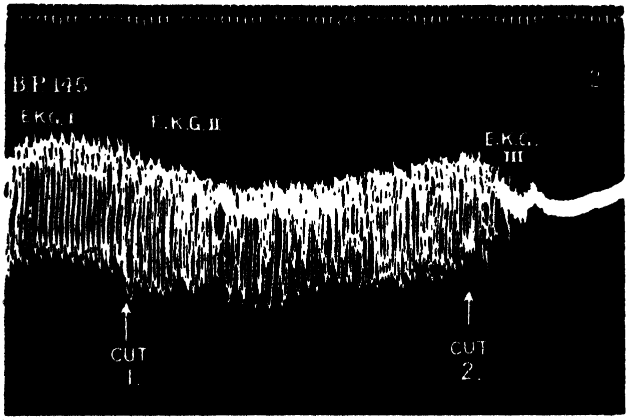
The knife used was a narrow single-edged paracentesis knife. with a blade of 1 centimetre in length and 2 millimetres in width. With this instrument it was possible to produce unilateral and also complete bilateral lesions. The knife had a sharp point and was guarded 1 centimetre from the point.

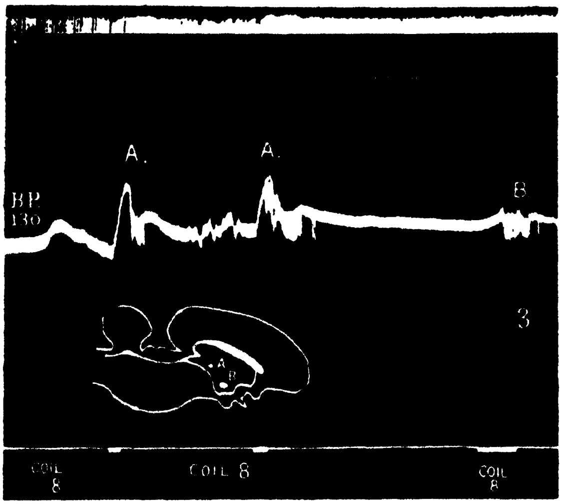
Using this instrument, or a piece of Gillette razor blade, 4 mm. wide and with a length of unguarded blade of 1 cm. (held firmly in a pair of hæmostatic forceps), stab wounds were made into the floor of the third ventricle at various places, with the same standard depth of wound. We found that a wound which passed from below, through or behind the mammillary bodies, and upwards in the direction of the anterior edge of the superior colliculus, was effective in abolishing the extrasystolic arrhythmia, provided that the width of the section was not less than 2 mm. from the middle line on each side and deep enough to reach the aqueduct of Sylvius. When the section had a width of this magnitude, and the animal was allowed to recover subsequently, there were no signs of motor disturbances (figs. 1 and 2, and Plate 23, figs. 1–4).

Unilateral lesions were of no avail in the abolition of the abnormal rhythm, although such lesions were identical in site and depth with the bilateral lesions.

The immediate effects of the section are of some interest. The extrasystoles disappeared and the blood pressure remained unchanged. The pupils contracted and did not react to light. In animals allowed to recover, the pupillary constriction was present, as a rule, only for the first 48 hours. The pupillary reflex then returned, but always remained sluggish.

From these experiments we concluded that the centres involved lay between the levels already indicated, and further, they lay close to the middle line below the thalamic commissure. Stab wounds into the brain anterior to the level of the effective stab wound had little effect on the extrasystolic rhythm. As a control (except in the recovery animals) a section anterior to the mammillary





TEXT-FIG. 2.—Blood pressure tracing, to show the effect of two sections into the hypothalamus at the levels *a* and *b* indicated in text-fig. 1.

TEXT-FIG. 3.—Blood pressure tracing, to show the effect of stimulation of small areas of the hypothalamus after hemi-decerebration; the left hemisphere is intact. The area A is situated close to the point of entrance of the aqueduct of Sylvius into the third ventricle. Area B is about 3 mm. above the origin of the infundibular recess and slightly posterior. Both these points are on the lateral wall of the third ventricle, to which the electrode is applied. The current used in the primary circuit was 3 volts and the coil was at 8 on the secondary coil scale. Extrasystoles are produced by stimulation at either of these points.

bodies was done in every experiment. We found that such sections did not disturb the arrhythmia nor affect in any way the blood pressure level.

Histological examination demonstrated that the lesion produced lay close

to the middle line and never extended as far laterally as the subthalamic body of Luys. From these observations a definite region was mapped out, with the following boundaries: anteriorly and superiorly, a line joining the anterior edge of the superior colliculus to the posterior edge of the optic chiasma (plane A, in fig. 1); posteriorly, a line joining the anterior edge of the superior colliculus to the posterior edge of the mammillary bodies (plane B, in fig. 1); laterally, an imaginary plane not greater than 3 or 4 mm. from the median sagittal plane. Within this region lie a centre or centres, the removal of which results in the abolition of extrasystoles (figs. 1 and 4).

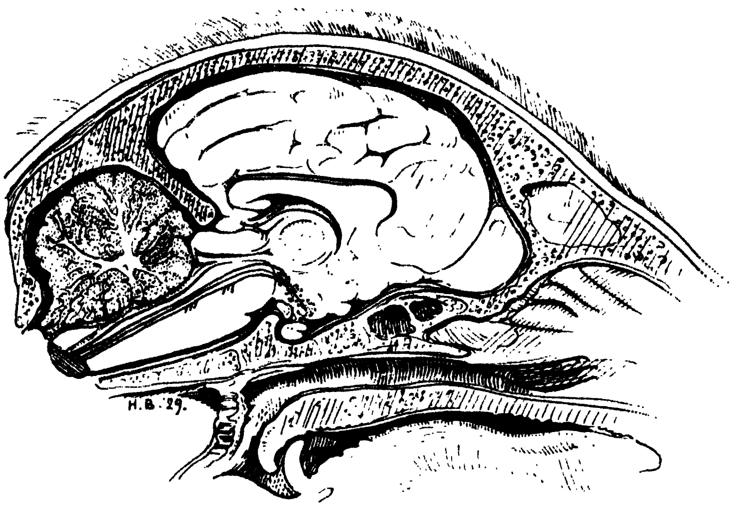
The Effects of Stimulation of the Hypothalamic Region under Chloroform Anæsthesia.—With the approximate localisation determined by the above experiments, we attempted to produce extrasystoles by a direct stimulation of the hypothalamus under chloroform anæsthesia during a period when such irregularities were absent. Two methods were tried: first, direct stimulation of the hypothalamus by the insertion of electrodes through the base of the third ventricle. This method was abandoned as the localisation of the site of stimulation was impossible and the results were therefore inconsistent.

A method was devised which consisted of a hemi-decerebration of the animal, performed under ether anæsthesia, some hours before a stimulation experiment was attempted. A careful dissection of the lateral wall of the third ventricle was carried out, to expose this region in its whole extent. The half-brain was removed posteriorly as far as the inferior colliculus. The animal was anæsthetised by chloroform during the stimulation. Using bipolar and unipolar electrodes the wall was stimulated, with the results seen in the tracings. Care was taken to prevent the spread of the current. This was checked by taking a simultaneous respiratory record and by stimulating other regions of the brain equidistant from the medulla. No effect was observed on the respiration upon hypothalamic stimulation.

Fig. 3 shows the sites of stimulation and fig. 4 the main cell groups of the region which are described later (p. 266). Area "A" (fig. 3) on stimulation causes a rise in blood pressure accompanied by extrasystoles after a short latent period. The fall in blood pressure is due to the presence of extrasystolic beats. This effect is shown very well in the second stimulation period in fig. 3. On stimulation of area "B" the blood pressure does not rise appreciably, as the stimulation is followed almost immediately by the extrasystolic beats, which mask the pressor effect. The electrocardiographic tracing taken during stimulation of area "A" is shown in Plate 23, fig. 5.

Stimulation of other regions of the hypothalamus did not produce rises in

blood pressure or extrasystoles. Only by stimulating along a line from about 3 mm. above the level of the infundibulum to the opening of the aqueduct of



TEXT-FIG. 4.—A median sagittal section of a cat's head, to show the position of the main posterior hypothalamic nuclei and their descending tracts as seen by projection on the median plane. The lowest mass corresponds to the premammillary nuclei, the middle to the posterior hypothalamic and the upper to the posterior periventricular nucleus. The diagram does not show the full posterior extent of the nuclei but only their general relations.

Sylvius was it possible to obtain the pressor effect and the extrasystolic arrhythmia.

Karplus and Kreidl (3) have shown that stimulation of the hypothalamus is followed by marked increases in blood pressure. They approached the region by removing the cerebral cortex and stimulating the region from above. The effect obtained did not depend upon the integrity of the adrenal glands or the pituitary. They showed that section of the splanchnics greatly diminished the pressor effect. Stimulation of the surrounding brain tissue was not effective in producing blood pressure changes.

Houssay and Mollinelli (4) have shown that situated around the ventral aspect of the third ventricle are masses of grey matter, the stimulation of which causes a great secretion of adrenaline. By careful technique they eliminated the possible spread of the current to the medulla. They showed that they could produce the same adrenaline secretion by stimulation of the floor of the fourth ventricle near the middle line.

These experiments, along with those of Cannon and Rapport (5), indicate

that there are probably two regions in the brain stem, stimulation of which is followed by pressor effects and adrenaline secretion—one of these is situated in the hypothalamus and the other in the bulb. It must not be forgotten that the effects obtained by bulbar stimulation may be due to a stimulation of a direct pathway from the hypothalamus to the spinal cord. The bulbar centre may be an important relay station in the projection pathway from the hypothalamus to the cord. These points will be discussed later.

The production of extrasystoles by stimulation of the hypothalamus is due partly to (*a*) stimulation of sympathetic pathways to the heart, and (*b*) stimulation of paths which are controlling the secretion of adrenaline. When the adrenal glands are removed stimulation of the hypothalamus still produces extrasystoles. The correlation of these stimulation experiments with the histology of the hypothalamus will be dealt with later.

The Effects of Sympathetic Stimulation and Paralysis or Extirpation on the Electrocardiogram.—It has been shown by Andrus and Martin (6) that sympathetic stimulation by the injection of adrenaline shortens the auriculo-ventricular conduction time. Rothberger and Winterberg (7) obtained the same results by faradic stimulation of the sympathetic and vagus nerves and by sympathetic stimulation after previous injection of barium chloride. We have demonstrated that section of the anterior roots of the second, third and fourth thoracic nerves on both sides increased the auriculo-ventricular conduction time and slowed the rate of the heart.

Stimulation of the hypothalamus diminished the A-V conduction time in the cat's heart from 0·08 second to 0·07 or 0·06 second, and at the same time increased the heart rate from 210 to 270 beats per minute. In another experiment the acceleration was from 120 to 180 beats per minute. Section of the hypothalamus on the other hand had the reverse effect, the A-V conduction time lengthening from 0·06 to 0·07 or 0·08 second and the heart rate decreasing from 240 to 210 or 180 to 120. These effects were pronounced when the vagi were intact but were rather less marked when the nerves were severed. The QRS complex showed little or no change with sympathetic stimulation or extirpation.

Levy (1) noted the development of ventricular extrasystoles when animals were anæsthetised with chloroform. Hume (8) observed the same type of arrhythmia after the injection of adrenaline into normal humans. Our observations demonstrate that ventricular extrasystoles occurring in the right and left ventricles can be elicited by stimulation of the hypothalamus or any part of the peripheral sympathetic mechanism of the heart. These abnormal

ventricular beats may be isolated or occur in groups, or the ventricles may show complete ventricular tachycardia. We have noted above the method whereby these abnormal beats may be abolished.

The Influence of the Vagus Nerve.—When extrasystoles have been produced in an animal by the inhalation of chloroform, and the vagi are sectioned, the extrasystoles still persist.

In considering the effects of vagal stimulation, there are two distinct effects to be considered. Strong stimulation of the central end of the vagus nerve (nerve sectioned) has been shown by Houssay and Mollinelli (9) to cause a reflex secretion of adrenaline. This is not the only effect, as we have shown that when the adrenals are removed central stimulation will still give rise to extrasystoles, if not previously present, when the animal is under chloroform. In this way, there seems to be no difference between the effects of vagus (central) stimulation from stimulation of the sciatic nerve or any other nerve, provided both vagi are sectioned. When one vagus is intact and the central end of the other is stimulated, slowing of the heart occurs, along with an abolition of extrasystoles.

Stimulation of the peripheral end of the vagus produces an entirely different effect from central stimulation. The heart rate immediately slows, with a complete disappearance of the extrasystoles. On cessation of the stimulus the irregularities reappear and continue. They can be obliterated again by restimulating.

II. *Survival Experiments.*

Greving (10), in his article on "Die vegetativen Zentren im Zwischenhirn," has summarised the literature on the structure of the hypothalamus and has added to it the results of a series of investigations of his own. He has described the distribution of the cell masses and the fibre tracts of the hypothalamus, but there are large gaps in our knowledge of the exact termination of the long projection tracts. There has been no direct anatomical proof of the connection between the hypothalamus and the lower parts of the central nervous system. In this paper we show the degenerations which result after lesions in the hypothalamus.

It is possible to deduce from Greving's work that the hypothalamus can be divided into two parts, which may differ considerably in function—

(*a*) Supra-optic infundibular complex.

(*b*) Tuber-paraventricular complex.

Fulton and Ingraham (11), in some recent work, would appear to have demonstrated that the anterior or supra-optic complex differs markedly from the

posterior or paraventricular portion. They have shown that lesions into the pre-chiasmatic hypothalamus produce profound changes in the behaviour of the animal. These changes partake of definite increase in the "wildness" of the animal. Hissing, growling and attempted biting were seen within 48 hours of operation, and persisted. In our animals, with lesions posterior to the level of the lesion used by Fulton and Ingraham (11), no such changes were observed. On the contrary, the opposite effect was seen. Animals which were inclined to be ferocious or wild became very docile and could be fed easily by hand. The change lasted until the animal was killed after 4 or 5 weeks.

Animals Used.—Cats were used in all recovery experiments. In all 16 animals were operated on and allowed to recover. In the early group of 8, bilateral lesions were attempted. Unilateral lesions were produced in the later animals.

The animals which were given bilateral lesions died within 10 days. The cause of death may be subdivided as follows :—

Meningitis and encephalitis	3 (within 3 days).
,, ,, ...	1 (after 8 days).
Pneumonia	2
Unknown	2

The 3 animals dying within 3 days of operation had extensive meningitis, due no doubt to faulty technique. The animal dying after 8 days was allowed to recover, without any attempt being made to repair the palate. The animal was examined on the sixth day, and under an anæsthetic the nasopharynx was washed out and sloughs removed. There is little doubt that the mere handling of the wound in the roof of the pharynx allowed organisms to enter non-infected areas. One of the animals dying from pneumonia had been given 50 c.c. of milk immediately after operation ; this was vomited some hours later and some of the fluid was aspirated into the lungs. The other animal also had an aspiration pneumonia. In this case the palate was not repaired, and on attempting to drink milk it was seen that the animal coughed violently after every drink. The two animals dying from "unknown" causes had no trace of pneumonic changes in the lungs and no sign of infection of the meninges. The wounds in the sphenoid were granulating well and were not badly infected. Each animal had lost over 500 grammes before death. One living to 9 days lost 550 grammes, and the other to 10 days lost 600 grammes.

In the later series, attempts were made to produce unilateral lesions. Eight

animals were used, but only two of these survived for a period greater than 28 days. The others died at various times and from various causes :—

Meningitis and encephalitis	2 (within 3 days).
Pneumonia	1 (within 48 hours).
Unknown causes	3 (after 10 days).

In all, 7 animals survived long enough to allow of observations being made on their nervous systems. The brain of one animal was not examined, as death had taken place at least 12 hours before. Of the six animals, two were examined by the Marchi method and the remainder by the Alzheimer-Mann technique.

Operative Methods for Recovery Experiments.

Anæsthesia.—During the early series of survival experiments we used di-allyl-barbituric acid as an anæsthetic. With this drug it was possible to obtain complete anæsthesia for a period of 36 hours with doses of 0·5–0·6 c.c. per kilo of body weight. The drug was used in the form of the proprietary preparation "Dial." It was injected into the peritoneal cavity.

We had hoped to gain many advantages by the use of this drug. It was not necessary, we found, to give supplementary doses after some time, as in the case of amytal or chloralose, nor was it necessary to expose a vein for the purposes of injection. We thought that the length of anæsthesia would prevent any unnecessary movement during the first 24 hours after the operation, and that the animal could be fed without struggling and the danger of hæmorrhage.

These advantages were more apparent than real. We found that as a rule it was better to have the animal recover quickly from the effects of the anæsthetic and to risk the possibility of hæmorrhage from the site of operation. Consequently we used 2 per cent. chloroform in the gas mixture or ether on a mask. Recovery from the anæsthetic took place within a few hours and the animal remained quiet for the first 3 days.

Operative Procedure.—We used the method of approach described by McLean (2). After the mouth cavity had been washed out with sterile saline, the whole region was painted with 1 per cent. mercurochrome in watery solution. The soft palate was split in the middle line from about 2 mm. posterior to the edge of the hard palate. The incision was continued backwards and stopped so as to leave about a centimetre of the soft palate intact at the posterior end. The edges were retracted either with small mastoid retractors or by means of sutures inserted through the soft palate near the cut edge. The edges of the wound were swabbed with mercurochrome and the nasal cavity washed out in the same manner as the mouth.

The muco-periosteum on the basi-sphenoid was split in the middle line and retracted as it was elevated from the bone. Using the posterior edge of the pterygoid lamina as a guide, a dental drill was used to bore away a small hole, about 5 mm. wide, in the middle line and with the posterior edge of the hole in line with the pterygoid lamina. When the dura and the underlying pituitary gland were seen through a thin bony plate, the most inferior part of the pituitary fossa was determined. At this inferior limit the bone was drilled through in the middle line, and with care the dura was exposed for about 4 or 5 mm. in the transverse plane. Bleeding was controlled by muscle compresses. The dura was next incised

in a longitudinal plane with a fine cataract knife. Any degree of traction on the dura led to bleeding. The pituitary gland bulged but little into the wound if the incision into the dura had been kept small (2 or 3 mm.). The risks of meningitis were lessened materially if the gland was retained within its dural coverings. The knife was then inserted into the pituitary fossa so as to strike its posterior wall. On reaching the dural roof of the fossa the knife was allowed to penetrate the dura just in front of this posterior edge and to travel into the brain substance, so that the total depth of incision was 1 cm. from the entrance into the dura covering the floor of the fossa. In this way it was possible to produce lesions which were in the plane necessary to abolish extrasystoles. Controls were made in the acute experiments, and it was found that such a stab wound as described above, and measuring but 2 mm. laterally from the middle line, would abolish extrasystoles in a cat under chloroform.

The wound in the palate was sewn up carefully, and although McLean recommended two layers of sutures in his animals we found that such duplication was unnecessary if the apposition of the suture line was accurate. The line must be watertight. Before being removed from the table, 50 c.c. of water or milk were placed in the stomach by a stomach tube. The animal was placed in a heated cage surrounded by water bottles, and on a wire floor so that urine could be collected.

Histological Technique.—Two methods were used to investigate the degenerations following lesions in the hypothalamus: (*a*) Marchi method, and (*b*) Alzheimer-Mann method. The latter technique is valuable, as changes in the degenerating axon can be detected at a very early stage (5 days). The normal axon stains blue and the degenerating, pink. As the stain is one for axon degeneration and not for myelin degeneration, it gives very accurate pictures of the changes in both medullated and non-medullated fibres.

Post-operative Observations. (1) *Sleep.*—We found as a constant feature that the animals were very lethargic and drowsy for 2 or 3 days after the operation. The lethargy persisted during the whole of the life of the animal after operation (31 days maximum). The day after operation they were very hard to rouse, and after being awakened invariably went off to sleep again.

(2) *Behaviour.*—It has been noted above that the animals became very quiet and docile after operation. In every case the "operated" animals sought the warmest part of the animal room when released from the cages for exercise. They crept close to radiators and huddled themselves against the pipes. Even in weather below zero we never observed pilo-erection on the side of the lesion, although the non-operated side showed the erection normally. In complete bilateral lesions, pilo-erection was always absent.

(3) *Temperature.*—No continuous record of temperature was kept on each animal. It was noted, however, that on the occasions on which a rectal temperature was taken it was about 36·5° C. or 37° C., when normal animals showed a temperature of 38° C.

(4) *Glycosuria.*—Every animal operated on had a definite glycosuria within 24 hours of operation. The amount observed in the urine at the end of 24 hours varied from 2·25 per cent. to 1·14 per cent. At the end of 48 hours, the amount was almost the same (2·30 per cent. to 1·5 per cent.). During the first week the glucose gradually fell, and reached a level varying from 0·4 per cent. to 0·7 per cent. After this time the fall continued, and in two animals, surviving over 4 weeks, the glucose was absent in measurable quantities after about 16 days. All traces had vanished by the twenty-fourth day. These animals had unilateral lesions. In one animal (cat 5 R) the blood sugar was 0·118 per cent. 1 hour after operation, 0·129 per cent. 2 hours after, and 0·138 per cent. 6 hours after. At the end of 24 hours it had risen to 0·492 per cent. Forty-eight hours after operation it had fallen to 0·210 per cent. The corresponding glycosuria at the end of 48 hours was 2·25 per cent.

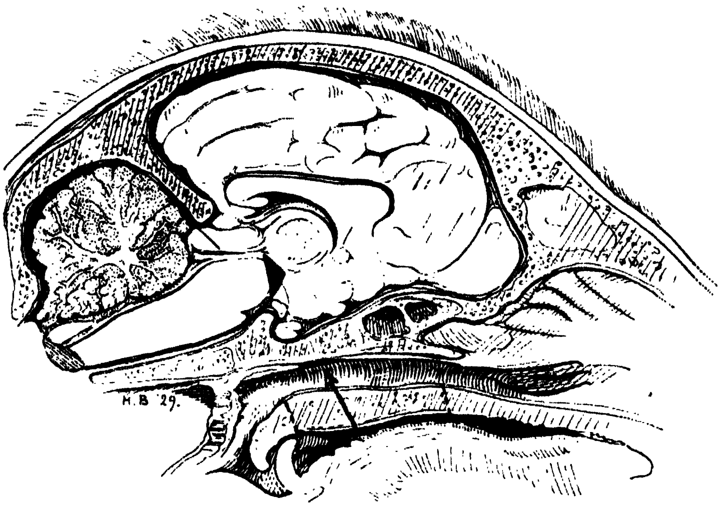
(5) *Albuminuria.*—Each animal showed a marked amount of albumin in the urine during the first 4 or 5 days after operation. In one animal (cat 38) the blood urea was 0·372 gramme per litre, and urea nitrogen 17·36 mg. per 100 c.c., 24 hours after operation. The albuminuria was most marked in those animals anæsthetised by di-allyl-barbituric acid.

(6) *Polyuria.*—No continuous records of the urine output were kept. The animals drank very much more than normally, consuming as much as 500 c.c. of milk per day during the first week. After this time the amount consumed was very much less.

(7) *Pupillary Changes.*—Immediately an effective section had been made, the pupil was found to be contracted. As the effect of the anæsthetic disappeared the contraction became more and more marked, until the pupil was slit-like. The pupil in this state did not react to light. After 2 or 3 days the contraction became less marked, and as it did so the reaction to light reappeared, but remained sluggish when compared with the normal eye.

The Site of the Experimental Lesions.—It has been pointed out that a lesion which is effective in abolishing extrasystoles lies in the colliculo-mammillary plane. In all the recovery experiments the lesions, whether unilateral or bilateral, were planned to lie in this plane. In text-fig. 5, the site of the lesion is plotted out on a median sagittal section. The lesion in every operation entered either through or behind the mammillary bodies. In width it extended as far as the lateral limit of the periventricular grey matter and about 1–1·5 mm. in an antero-posterior direction. Fig. 5 and Plate 24, figs. 1 and 2, show the extent of the lesion in cat 16 R. In one animal the lesion penetrated deeper

than the grey matter and damaged the tract of Meynert. Degenerated fibres were seen to pass into the posterior commissure.



TEXT-FIG. 5.—A median sagittal section, to show the position of the unilateral lesion in cat 16 R. The arrow indicates the point where the base of the skull is drilled to obtain access to the pituitary fossa. The broken lines across the palate show the boundaries of the incision through this structure.

When an attempt is made to correlate the site of experimental lesions in the hypothalamus with the cell masses there, one is faced with some difficulty in terminology. At least three nomenclatures for cell masses have been proposed. Winkler and Potter (12), Gurdjian (13) and Greving (10).

Gurdjian, working on the brain of the albino rat, has described the hypothalamic nuclei and their connections in this animal, in such a way that there is little difficulty in applying it to the brain of the cat. Winkler and Potter described the cat's brain, but their study of the hypothalamus was not complete. Greving, in human material, described hypothalamic nuclei under different names to those adopted by Gurdjian. In general, Gurdjian's description from a study of toluidin blue preparations fits fairly closely the distribution of the nuclear masses in the cat.

The Nuclear Masses.—It has been pointed out above that the hypothalamic nuclei are divided probably into two groups—a supra-optic-infundibular group and a tuber-paraventricular group. Greving emphasises the intimate connection of the supra-optic group with the hypophysis, and although there is a connection between the hypophysis and the posterior hypothalamic group, it is not so extensive as the former. Gurdjian points out that the cell masses

associated with the periventricular fibre tracts lie in the more postero-dorsal region of the hypothalamus. Such a condition would seem to be present in the cat. Stimulation, to be effective in the production of extrasystoles, must be carried out in the posterior hypothalamic region. Again, the major part of the supra-optic group of nuclei are cut off when a section is made from the anterior edge of the superior colliculus to the caudal edge of the optic chiasma. Such a section does not abolish extrasystoles, as we have pointed out before.

When the hypothalamic nuclei are examined posterior to this line of section, the cell masses can be subdivided into the following nuclei :—

Medial.

1. Nucleus premammillaris dorsalis.
2. Nucleus premammillaris ventralis.
3. Nucleus hypothalamicus posterior.
4. Nucleus hypothalamicus periventricularis posterior.

Lateral.

5. Nucleus hypothalamicus lateralis.

These nuclei correspond closely to those described by Gurdjian in the rat. The lateral nucleus need not concern us, as we have not injured it in any of our experiments.

The four nuclei described above are situated on the lateral wall of the third ventricle, and partly on its floor posterior to the infundibulum. The premammillary nuclei correspond closely to the nuclei tuberis and the nucleus mammillo-infundibularis described by Greving. The anterior part of the nucleus periventricularis posterior also extends into the tuber region. The nucleus hypothalamicus posterior can be traced caudally into the tegmentum of the mid-brain, and so corresponds with the nucleus of the same name described by Gurdjian and the nucleus reticularis of the hypothalamus described by Greving. The nucleus periventricularis posterior corresponds to the nucleus paraventricularis of Greving, and partly to the nucleus medialis hypothalamicus of Winkler and Potter.

Each of these nuclear masses sends fibres into the periventricular tracts. Consequently, on section of these tracts by a lesion placed in the plane from the posterior edge of the mammillary body to the anterior edge of the superior colliculus, the whole group is severed from the lower centres (text-fig. 5).

Pathological Changes at the Site of the Lesion.—In all the animals investigated,

the lesion was found to be in direct continuity with the third ventricle. The lesion was filled with degenerated tissue which was in process of being absorbed by phagocytes. These cells were heavily stained by the osmic acid in the section (Plate 24, figs. 1 and 2). We did not investigate the glial reaction to the lesion.

The Degenerated Tracts after Experimental Lesions.—In two animals degenerated fibres were traced anterior to the site of the lesion. In Plate 24, fig. 4, degenerated fibres are seen crossing the median plane in the supra-optic decussation. These fibres, when traced, pass laterally and enter the medial forebrain bundle, making their way to the region of the pyriform lobe. The degeneration may be retrograde. If the degeneration is retrograde and the termination of the fibres is, as seems most probable, in the pyriform area, then the hypothalamus at the site of the lesion is probably in close relation to the olfactory cortex.

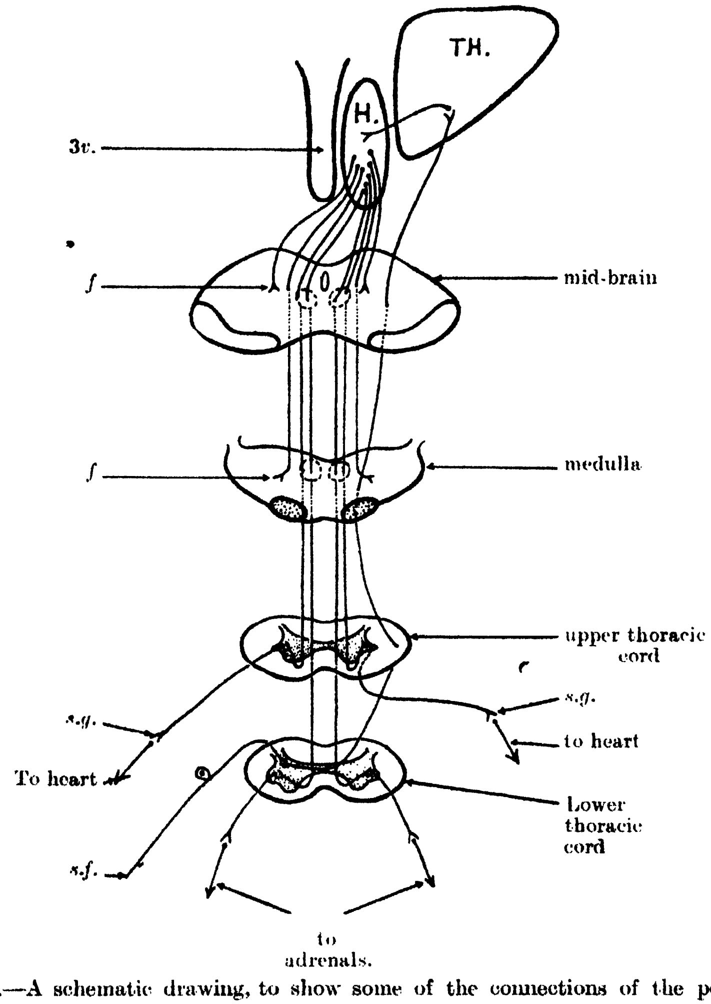
No degenerated fibres have been traced into the thalamus or laterally into the subthalamic nuclei. Ventral to the lesion (Plate 24, fig. 1) degenerated fibres can be seen which cross the mid-line in the anterior part of the decussation of Forel. On tracing these fibres, they commence on the more ventral aspect of the lesion, and after crossing the mid-line pass down as periventricular fibres on the ventral and lateral aspects of the grey matter of the cerebral aqueduct. There is little doubt that the presence of this decussation partly explains the invariable presence of contra-lateral fibres in sections caudal to the lesion.

Fibres arising from the caudal part of the lesion pass directly along the outer surface of the central grey matter and so into the mid-brain. They are fine medullated fibres with a few larger fibres admixed. They do not form large bundles but are spread out in a thin layer. The fibres which have crossed the mid-line in the decussation of Forel are cut as they arch upwards along the lateral wall of the posterior part of the third ventricle.

When the lesion has been placed so as to avoid damage to the mammillary body, the mammillo-thalamic tract is intact. In one animal the lesion passed through the mammillary body on one side and degeneration of this tract was seen. All the pure hypothalamic degeneration lies medial to the tract and to the anterior pillars of the fornix.

As the fibres pass caudally they pass on the medial side of the fasciculus retroflexus of Meynert. They enter the mid-brain by passing ventral to the posterior commissure fibres. Some of the fibres immediately after entering the mid-brain are seen to pass laterally and to enter the ventral aspect of the

superior colliculus, where they apparently end. The remainder commence to arch ventrally, except the more lateral fibres, which enter the dorso-lateral part of the mid-brain tegmentum in the reticular formation (fig. 6). In the mid-brain the fibres are in close association with some of the fibres of the mesencephalic root of the fifth nerve, but they are easily distinguished from the fifth nerve fibres by their size.



TEXT-FIG. 6.—A schematic drawing, to show some of the connections of the posterior hypothalamic nuclei. Some fibres from the hypothalamic nuclei are ending in the formatio reticularis of the mid-brain and medulla. Others enter the anterior columns of white matter of the cord and end in the lateral columns of grey matter. A hypothetical connection is inserted between the thalamus and hypothalamus.

3*v*., third ventricle ; *f.f.*, fibres ending in formatic reticularis ; *S.t.*, spino-thalamic tract ; *s.g.*, stellate ganglion ; *s.f.*, sensory nerve.

At the level of the inferior colliculus most of the degenerated fibres are concentrated on the deep surface of the grey matter of the floor of the fourth ventricle. Near the middle line they become mixed up with the large fibres of the posterior longitudinal bundle. The more lateral fibres are very much scattered in the dorsal reticular formation. One gets the impression that at or about this region a further partial decussation takes place, as the number of contra-lateral fibres appears to become much greater (Plate 2, figs. 3 and 5).

The course throughout the pons and medulla remains unchanged from that in the mid-brain with the exception that the fibres become more and more concentrated towards the median plane. In the lower medulla, below the decussation of the pyramids, the fibres take up a ventral position with the vestibulo-spinal fibres and so become scattered in the fasciculus proprius anterior, and to a lesser extent in the more lateral part of the ventral white matter.

In the upper thoracic cord the number of fibres begin to diminish and many make their way into the grey matter of the lateral horn. In this they apparently descend for a little distance, as fibres cut transversely are frequently seen in this position (Plate 2, fig. 6). The inflow into the lateral horn of grey matter continues throughout the whole thoracic region and caudally into the upper lumbar segments. In the third lumbar and fourth lumbar segments the numbar of fibres is exceedingly small, and in one animal at this level (lumbar 4) none were observed.

The degenerated fibres are small and finely medullated. The Alzheimer-Mann technique shows that the Marchi pictures are not altogether accurate, since many of the periventricular fibres as far as the mid-brain have no myelin sheaths. This technique demonstrates that the total axon degeneration is considerably greater than that shown by the Marchi method. The relative position and the termination of the fibres is identical by both methods of investigation.

Stimulation Experiments.—We have shown that stimulation of a sharply localised area of the hypothalamus will give rise to a cardiac arrhythmia under chloroform. The area stimulated lay in the posterior region of the hypothalamus along a line from about 3 mm. dorso-caudal to the infundibulum to the opening of the aqueduct. Such an area would correspond closely to the situation of the nucleus periventricularis posterior and the nucleus hypothalamicus posterior. We noticed that stimulation of the tuber cinereum, taking care to avoid spread of the current, produced no pressor effects or extrasystoles (fig. 4).

Brown and Sherrington (14) working on decerebrate preparations have shown that pilo-motor effects can be obtained by stimulation of certain regions of the mid-brain. When the locus of their stimulation is examined, it is seen that it lies in the fibre pathway of the hypothalamic nuclei.

III. *Discussion.*

It has gradually become known that the conception of Langley (15) and Gaskell (16) of the anatomy of the sympathetic nervous system is incomplete. It is to-day realised that in the hypothalamus, and possibly in other parts of the upper brain stem, there exist a number of nuclei which govern to a great extent the reactions of this system. Already a large number of sympathetic phenomena have been shown to be more or less dependent on the proper functioning of these areas. Among these are certain aspects of carbohydrate metabolism (17, 18, 19, 20), temperature regulation (21, 22, 23, 24, 25), pilo-motor and ocular phenomena (14, 26), pressor effects (3), and (as is to be expected) the secretion of the adrenal medulla (4). The recent work of Bard (27) and of Fulton and Ingraham (11) has illustrated the influence of cerebral centres upon these sympathetic nuclei. All this work has opened up a great field of study upon the correlation of all forms of sympathetic activity in these areas of the brain stem.

Levy's work showed that the development of cardiac irregularities in an animal under chloroform is dependent upon the intactness of the lower sympathetic neurones. Our work we believe has illustrated the fact that breaking the nervous connection between the hypothalamus and the spinal cord has a similar effect.

In animals surviving unilateral puncture of the hypothalamus the nuclei involved in the lesions are confined to the posterior part of the hypothalamus. Bailey and Bremer (20) have shown that the nuclei around the tuber cinereum are concerned with the production of diabetes insipidus. We have not been able to produce extrasystoles by stimulation of this region. Posterior to the tuber, and between it and the opening of the aqueduct into the third ventricle, stimulation of the lateral wall of the ventricle gives rise to extrasystoles under chloroform anæsthesia, and to pressor effects when extrasystoles are not immediately produced. Such a region corresponds to the situation of the nucleus hypothalamicus posterior and the nucleus hypothalamicus periventricularis posterior of Gurdjian, and partly to the substantia reticularis hypothalami and the posterior part of the nucleus paraventricularis of Greving.

A study of the degeneration shows that a lesion in the posterior part of the hypothalamus, lying medial to the column of the fornix and the mammillo-thalamic tract, produces degenerative changes in fibres passing downwards into the thoracic and upper lumbar region. These fibres are partly crossed but mainly uncrossed; they descend alongside the lateral and ventral boundaries of the central grey matter of the mid-brain, and eventually become concentrated into the more ventral part of the posterior longitudinal bundle and the dorsal part of the reticular formation of the medulla. In the mid-brain, pons and medulla, some of the fibres seem to pass into and end in the dorso-medial part of the formatio reticularis.

The hypothalamic nuclei which we have described above are associated apparently with the medial forebrain bundle, and probably with the pyriform lobe, but the latter fact is not yet certain. The partial decussation of the descending fibre tracts takes place in many regions—in the supra-mammillary part of the decussation of Forel and in the upper part of the brain stem.

The hypothalamus does not send fibres laterally into the thalamus. It has been pointed out above that a section from the anterior edge of the superior colliculus to the optic chiasma leaves no part of the globus pallidus in the intact part of the brain. Such a section does not abolish extrasystoles or interfere with the reflex production of these abnormal beats by sciatic stimulation; consequently it would appear that the hypothalamic nuclei described above are connected directly to the thalamus, and not indirectly through the corpus striatum.

The fibres which pass into the formatio reticularis may assist in the formation of other pathways down into the spinal cord. The existence of such parallel paths (Papez (28)) would explain the persistence of some sympathetic effects after decerebration, since Cannon and Rapport (5) demonstrated reflex acceleration of the heart after decerebration at the level of the inferior colliculus.

The Marchi method does not offer a conclusive demonstration of the exact number or mode of termination of the degenerated neurones. These have been traced into the intermedio-lateral column of grey matter, and there they apparently end. It would seem, then, that there are some nuclei in the diencephalon which send direct fibres to the columns of grey matter in the spinal cord which are known to give rise to preganglionic sympathetic fibres. There are, no doubt, lower bulbar centres which may modify or control the lower neurones in the spinal cord, but our experimental work would seem to show that there is, in the sympathetic system, a cephalisation of control analogous to that of the cerebro-spinal motor system.

The work cited here, along with that of Houssay and Mollinelli (4) and Karplus and Kreidl (3), would indicate that in the hypothalamus there are centres for the control of adrenaline secretion. Furthermore the experiments of Zuntz and La Barre (29) indicate that the blood-sugar level in the cerebral circulation determines the level of blood-sugar in the body generally. The adrenals and the islet tissue of the pancreas play important rôles in this mechanism. It would be of great interest to determine whether brain sections, such as we have described, would abolish this effect; for if this is so then the paramount importance of the hypothalamic nuclei in the regulation of carbohydrate metabolism would be established.

As we have shown in this paper, the sympathetic centres of the hypothalamus do influence heart rate. We have pointed out above that they can control carbohydrate metabolism. The work of Smith (30) and many others has demonstrated that disturbance of fat metabolism, water and salt metabolism (diabetes insipidus) also have central representation in this region. Certain emotional states are accompanied by signs of sympathetic activity, cardiac acceleration, pupillary dilatation, etc. (Penfield (31)).

There can be no doubt whatever that the sympathetic phenomena of such emotional states must be initiated and controlled by a mechanism at a higher anatomical level than the spinal cord. The work of Bard (27) is particularly interesting in this respect, since he has shown that spontaneous sham rage can no longer occur in an animal deprived of its hypothalamus by a brain section exactly similar to that which we have used to abolish extrasystoles. Conversely, Fulton and Ingraham (11) have produced a chronic state of sympathetic hyperactivity by liberating these hypothalamic nuclei from their cerebral connections.

IV. *Summary.*

1. It has been shown that extrasystoles induced by chloroform anæsthesia may be abolished by section of the hypothalamus in a plane between the anterior edge of the colliculi and the posterior edge of the pituitary fossa.

2. Stimulation of the posterior part of the lateral wall of the third ventricle produced an extrasystolic cardiac arrhythmia in an animal under chloroform anæsthesia.

3. Experimental lesions involving certain nuclei of the posterior part of the hypothalamus are followed by descending degeneration into the spinal cord.

4. The fibre tracts pass partly into the formatio reticularis of the brain stem and partly into the intermediolateral columns of grey matter in the thoracic and upper lumbar part of the spinal cord.

5. The relation of these fibre tracts and hypothalamic nuclei to various sympathetic phenomena is discussed, and evidence brought forward to demonstrate that the descending hypothalamo-spinal tracts play an important part in the control of the bulbar and spinal sympathetic nuclei.

We have to thank the Trustees of the Cooper Fund of McGill University for generous grants in aid of this research. We wish to acknowledge the help of Dr. W. V. Cone, of the Department of Neurosurgery of the Royal Victoria Hospital, in the preparation of our histological material. He introduced us to the Alzheimer-Mann technique, and carried out personally many of the preparations of our material.

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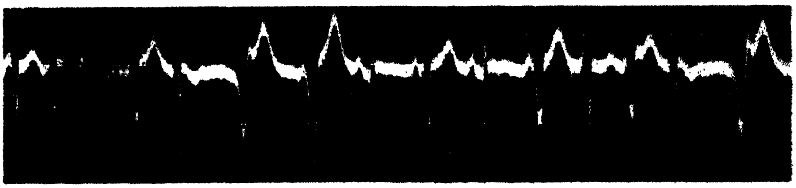

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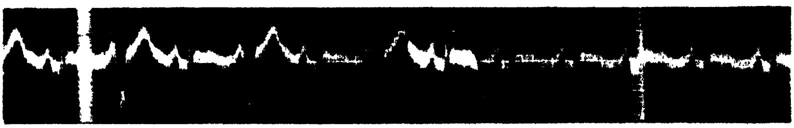

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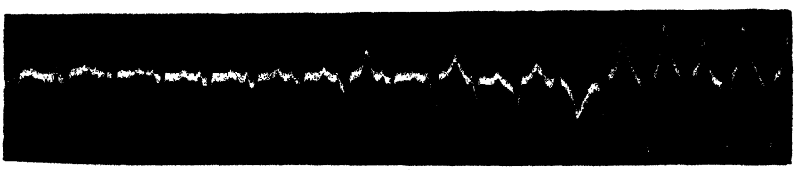

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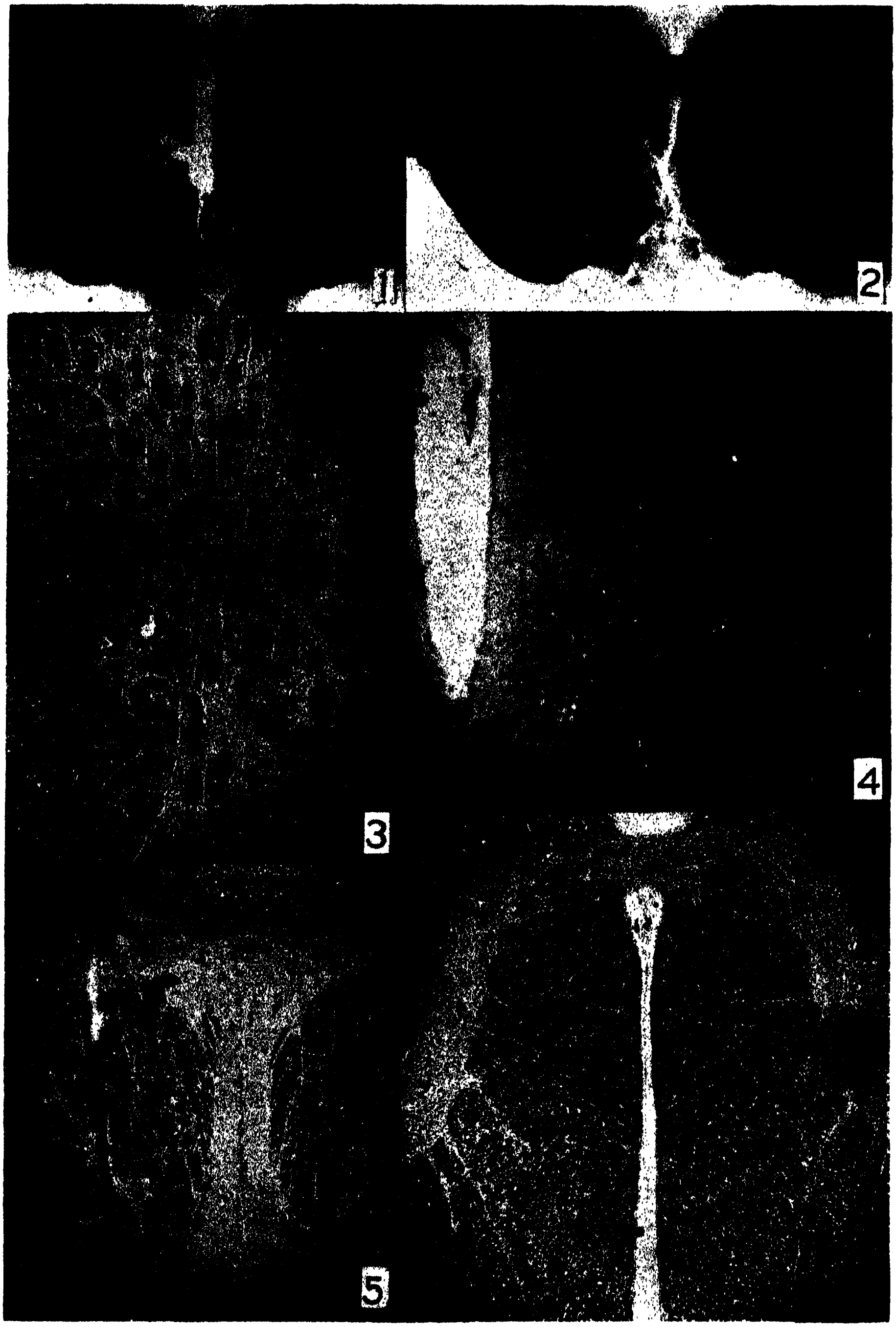
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VI. EXPLANATION OF PLATES.

Plate 23.

Fig. 1.—Electrocardiographic tracing (lead II) of a cat under chloroform anæsthesia, showing ventricular extrasystoles. Auricular rate 240 per minute.

Fig. 2.—Same cat after section had been made into the anterior part of the hypothalamus. The arrhythmia remains unchanged.

Fig. 3.—Same cat after section into the hypothalamus in the plane joining the anterior edge of the colliculi to the posterior edge of the pituitary fossa. The extrasystoles are fading out and normal rhythm is appearing. Auricular rate 180 per minute.

Fig. 4.—Same cat 1 hour after the effective section, to show the persistence of regular rhythm.

Fig. 5.—Electrocardiographic tracing, to show the production of extrasystoles by stimulation of the hypothalamus.

Plate 24.

Fig. 1.—Transverse section through the brain of cat 16 R (4040). The lesion is unilateral. Degenerated fibres can be seen crossing the middle line in the white matter dorsal to the mammillary bodies.

Fig. 2.—Transverse section through the brain of cat 16 R (4040) to show the caudal part of the lesion. The section passes through the anterior part of the interpeduncular space.

Fig. 3.—Transverse section through the lower pons in cat 16 R (4040). Degenerated fibres are present in the posterior longitudinal bundle and in the adjoining reticular formation. The posterior longitudinal bundle is on the left of the figure.

Fig. 4.—Transverse section at the level of the optic chiasma (cat 16 R—4040) to show degenerated fibres which have crossed in the supra-optic commissure.

Fig. 5.—Transverse section through the posterior longitudinal bundles in the upper pons in cat 16 R (4040) to show the small number of degenerated fibres on the contralateral side to the lesion.

Fig. 6.—The distribution of degenerated fibres in the white matter on either side of the anterior median fissure, at the level of the fourth thoracic segment.

Cat 16R (4040) was sacrificed on the twenty-third day after operation. The sections illustrated above were stained by the Marchi method.

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Visual Acuity in Light of Different Colours.

By H. E. Roaf.

(Communicated by Sir Charles Sherrington, O.M., F.R.S.—Received March 6, 1930.)

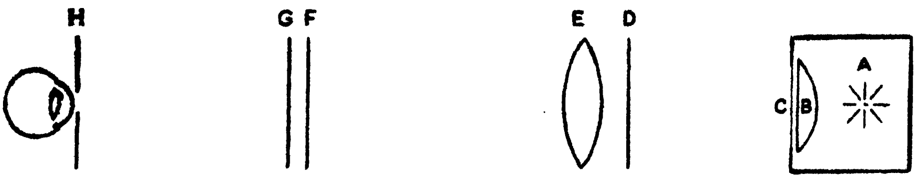
(From the Department of Physiology, London Hospital Medical College.)

Introduction.

Previous work on visual acuity has shown that there is a different law relating intensity of illumination to acuity of vision for lights of low and of high intensities. Hecht has recently discussed this problem,(1) basing his discussion on the measurements of König (2). König obtained poor acuity of vision with " blue " light. In view of the importance to the study of colour vision of any method of quantitative measurement, the following experiments were undertaken.

Method.

A bright light (A, fig. 1) was concentrated by means of a condenser (B) on a colour filter (C) and the light was concentrated on a ground-glass screen (F)



Plan of apparatus.

Elevation of test type and shutter.

Fig. 1. A = light. B = condenser. C = colour screen. D = shutter. E = lens. F = ground glass. G = test type. H = Eye screen.

by means of a lens (E). Close to the lens was a shutter (D) consisting of two sectors, each with three apertures of 60°, so that when the two sectors were superimposed with the open parts opposite to each other half the lens was

exposed to light, but when one shutter was rotated so that the opaque parts of it coincided with the apertures of the other, all the light ought to have been cut off. As a matter of fact some stray light found its way past when the shutter was supposed to be entirely closed, but the amount so passing was slight.

The movable sector was turned by a handle which passed across a millimetre scale. When the shutter was fully open the reading on the scale was 170 and when closed 0; therefore there was a range of illumination available from 0 to 170, and the amount of light passing through was directly proportional to the reading in millimetres.

Beyond the ground-glass screen was a carrier (G) in which lantern slides could be placed. These were viewed by one eye through a hole in a screen (H). There was a chin-rest beyond H, so that the observing eye was 54 cms. from the lantern slide, the other eye being obscured by the screen H.

Most of the observations have been made using photographs of test types, but a few readings have been taken with a broken circle or interrupted lines. The procedure was to start with the shutter, closed so far that the type could not be read, and for the subject to open it until the type was legible. Several independent settings of the shutter were made for each specimen of type and then another size of type was put in the carrier. In this way for each colour filter an average reading of the shutter was obtained for each of the series of test types.

The test types consisted of black letters on a transparent background, but a few observations were made using transparent letters on a dark background. The latter were found to be less easily read than the former, and were not used for more than a few subsidiary observations. In many observations an artificial pupil was used, in order to prevent the pupillary change from decreasing the relative amount of light with the higher intensities, but the pupil did not alter the general character of the result and was a disadvantage because it added to the difficulties of obtaining measurements, especially with untrained subjects.

The colour screens were examined visually and photographically. In addition to spectroscopic examination, a light was looked at through the superimposed screens to determine whether there was any overlap in the transmission. Examination of a hundred candle-power "point-o-lite" lamp through the "red" + "green" filters showed no trace of light; through "red" + "blue" filters a faint violet spot of light was visible, and through "blue" + "green" filters the target was visible as a pale blue-green disc.

With very high intensities it is possible that other wave-lengths than those measured below might be raised to threshold value.

Photographically, screen 8 (red) showed transmission from about 6600 Å. to the red end of the visible spectrum; screen 10 (green) showed transmission from about 4960 Å. to 5560 Å., and the screen 12 (blue) from about 4170 Å. to 4790 Å. These values vary with the intensity of the light and the duration of the exposure.

When we wish to compare the physical properties of the light with its physiological effect it is useful to have some simple comparison between the wave-lengths. The maximum amount of transmitted light will be in the central region of the band, therefore we can consider screen 8 as having the effect of a group of rays of about 6800 Å., screen 10 that of about 5300 Å., and screen 12 that of about 4500 Å. These are almost in the ratio of 6 : 4·7 : 4. The ratios are of importance in relation to the effect of the wave-lengths on resolving power of an optical system, but any overlap in transmission with green and blue is to be remembered when discussing the limitation of acuity of vision with screen 12 (blue).

Results.

Most of the records were made by two individuals, but a number of other subjects have been examined. In all cases the results are comparable, namely, that the acuity of vision is dependent on the intensity of light; but with screen 12 (blue) a much lower acuity is obtained than with screens 8 and 10. It seems unnecessary to record all experiments which have been made, therefore only some of the results are given in the tables. These experiments agree with the detailed observations of König (2).

Table I.

May 8, 1929. J.L.B. using a 2·08 mm. artificial pupil.

Screen 8 (red).		Screen 10 (green).		Screen 12 (blue).	
Test type.	Sector.	Test type.	Sector.	Test type.	Sector.
m.	mm.	m.	mm.	m.	mm.
Light visible	1	Light visible	0	Light visible	0
9	10	9	4	9	13
6	11	6	4	6	26
2	20	2	12	2	Not readable*
1	51	1	39		
0·75	111	0·75	65		
0·5	Not readable*	0·5	Not readable*		

* "Not readable" in these tables means that the letters could not be read even with the sector fully open at 170 mm.

Table I—(continued).

May 8, 1929. H.E.R., 2·08 mm. artificial pupil.

Screen 8 (red).		Screen 10 (green).		Screen 12 (blue).	
Test type.	Sector.	Test type.	Sector.	Test type.	Sector.
m.	mm.	m.	mm.	m.	mm.
Light visible	2	Light visible	0	Light visible	0
9	9	9	5	9	29
6	9	6	6	6	58
2	22	2	23	2	Not readable
1	51	1	56		
0·75	64	0·75	75		
0·5	Not readable	0·5	Not readable		

May 24, 1929. H.E.R. Without artificial pupil.

Screen 8 (red).		Screen 10 (green).		Screen 12 (blue).	
Test type.	Sector.	Test type.	Sector.	Test Type.	Sector.
m.	mm.	m.	mm.	m.	mm.
Light visible	2	Light visible	0	Light visible	0
9	10	9	7	9	26
6	12	6	5	6	33
2	27	2	15	2	Not readable
1	71	1	45		
0·75	105	0·75	86		
9·5	Not readable	0·5	Not readable		

July 15, 1929. H.E.R. Without artificial pupil.

Screen 8 (red).		Screen 10 (green).		Screen 12 (blue).	
Test type.	Sector.	Test type.	Sector.	Test type.	Sector.
m.	mm.	m.	mm.	m.	mm.
Light visible	1	Light visible	0	Light visible	0
8	17	8	8	8	64
3	43	3	28	3	Not readable with or without lenses.
1·6	61	1·6	63		
1·1	164	1·1	75		
0·75	Not readable	0·75	Not readable		

(In this experiment transparent letters on a dark back ground were used.)

Table I—(continued).

August 17, 1929. C.W. Without artificial pupil.

Screen 8 (red).		Screen 10 (green).		Screen 12 (blue).	
Test type.	Sector.	Test type.	Sector.	Test type.	Sector.
m.	mm.	m.	mm.	m.	mm.
Light visible	2	Light visible	0	Light visible	0
9	9	9	3	9	11
6	7	6	5	6	20
2	16	2	11	2	Not readable
1	28	1	21		
0·75	59	0·75	35		
0·5	Not readable	0·5	Not certain but some letters visible at 115 mm.		

January 29, 1930. H.E.R. No artificial pupil.

Screen 8 (red).		Screen 10 (green).		Screen 12 (blue) —1 D lens used.	
Test type.	Sector.	Test type.	Sector.	Test type.	Sector.
m.	mm.	m.	mm.	m.	mm.
Light visible	1	Light visible	0	Light visible	0
9	4	9	1	9	7
6	3	6	1	6	18
2	7	2	5	5	28
1	36	1	20	4	68
0·75	93	0·75	31	3	83
0·5	Not readable	0·5	Not readable	2	Not readable

January 29, 1930. H.F. No artificial pupil.

Screen 8 (red).		Screen 10 (green).		Screen 12 (blue) —1 D lens used.	
Test type.	Sector.	Test type.	Sector.	Test type.	Sector.
m.	mm.	m.	mm.	m.	mm.
Light visible	3	Light visible	0	Light visible	0
9	9	9	3	9	21
6	7	6	3	6	33
2	24	2	7	5	37
1	118	1	12	4	58
0·75	Not readable	0·75	63	3	90
		0·5	Not readable	2	Not readable

Table I—(continued).

February 5, 1930. H.E.R. No artificial pupil.

Screen 8 (red).		Screen 10 (green).		Screen 12 (blue) —1 D lens used.	
Test type.	Sector.	Test type.	Sector.	Test type.	Sector.
m.	mm.	m.	mm.	m.	mm.
Light visible	1	Light visible	0	Light visible	0
9	4	9	2	9	6
6	4	6	3	6	11
2	9	2	7	5	27
1	33	1	23	4	41
0·75	54	0·75	40	3	100
0·5	Not readable	0·5	Not readable	2	Not readable

February 5, 1930. H.F. No artificial pupil.

Screen 8 (red).		Screen 10 (green).		Screen 12 (blue) —1 D lens used.	
Test type.	Sector.	Test type.	Sector.	Test type.	Sector.
m.	mm.	m.	mm.	m.	mm.
Light visible	3	Light visible	0	Light visible	0
9	7	9	3	9	14
6	6	6	3	6	15
2	16	2	6	5	18
1	60	1	17	4	33
0·75	86	0·75	34	3	42
0·5	Not readable	0·5	Not readable	2	87
				1	Not readable

February 18, 1930. C.E.B. No artificial pupil.

Screen 8 (red).		Screen 10 (green).		Screen 12 (blue) —1 D lens used.	
Test type.	Sector.	Test type.	Sector.	Test type.	Sector.
m.	mm.	m.	mm.	m.	mm.
Light visible	2	Light visible	0	Light visible	0
9	13	9	5	9	17
6	14	6	6	6	20
2	21	2	13	5	43
1	68	1	19	4	60
0·75	133	0·75	28	3	60
0·5	Not readable	0·5	67	2	Not readable

König made his observations on the relation of form vision to intensity of illumination over a wide range of values. He used black letters on a coloured background and examined them by light which had passed through colour filters, but he does not state the wave-length transmission of his filters. He states that the restriction of visual acuity for "blue" is due to the greater spherical aberration with that light. His figures for the logarithms of the maximum intensities used are 4·61 for "red" light, 5·11 for "green," and 5·80 for "blue," whilst the corresponding acuities are given as 1·740, 1·633 and 0·640 respectively.

The intensities of the different coloured lights given above are those of the light before it had passed through the filters. With reference to the form vision of a "totally colour blind" individual, König remarks that measurements were limited by violent dazzling (*heftige Blendung*) by the higher intensities. The relative intensities of the lights must be discussed later in this paper.

Discussion.

The results clearly show that visual acuity is much less with short than with medium or long wave-lengths. This is not due to difference in refrangibility of the rays. On purely optical grounds the resolving power should be greater for blue than for red, in the ratio of about 3 to 2.* This is why the term resolving power is not used in describing the results. The difference in refrangibility (chromatic aberration) might lead to failure to focus accurately on the retina.

It is probable that accommodation is regulated by the predominant wave-lengths. Thus within the range of accommodation, each colour might be in focus when viewed by itself. In order, however, to obviate any defect due to failure to focus properly, special experiments were carried out, with lenses in front of the eye used for reading the letters. With some subjects slightly better results were obtained with a —1 dioptre lens when using screen 12, thus indicating that with relaxed accommodation there was a tendency for "blue" light to focus in front of the retina, but with other subjects there was no difference.

The acuity in "red" light was not improved by either positive or negative lenses. The figures quoted in Table I were the best obtainable with correction by lenses, but in order to show that the differences due to failure to focus are small compared with the differences in acuity due to the wave-length, the following results from experiments are given.

* See above, p. 278.

Table II.

May 31, 1929. J.L.B. No artificial pupil.

Screen 8 (red).			Screen 10 (green).			Screen 12 (blue).		
Test type.	Lens.	Sector.	Test type.	Lens.	Sector.	Test type.	Lens.	Sector.
m.		mm.	m.		mm.	m.		mm.
9	−1	18	9	−1	13	9	−1	40
9	None	*11*	9	None	*5*	9	None	*18*
9	+1	17	9	+1	7	9	+1	73
0·75	−1	Not readable	0·75	−1	Not readable	2	−1	Not readable
0·75	None	*107*	0·75	None	*62*	2	None	Doubtful at 110 mm.
0·75	+1	111	0·75	+1	81	2	+1	Not readable

December 17, 1929. H.E.R. No artificial pupil.

Screen 8 (red).			Screen 10 (green).			Screen 12 (blue).		
Test type.	Lens.	Sector.	Test type.	Lens.	Sector.	Test type.	Lens.	Sector.
m.		mm.	m.		mm.	m.		mm.
9	−1	7	9	−2	3	9	−2	25
9	None	*4·5*	9	−1	2·5	9	−1	*8*
9	+1	6	9	None	*2·0*	9	None	16
6	−1	6	9	+1	4	6	−2	36
6	None	*4*	6	−2	4·5	6	−1	*16*
6	+1	5	6	−1	*2·5*	6	None	21
2	−1	45	6	None	*2·5*	6	+1	85
2	None	*10*	6	+1	5	2	−2	Not readable
2	+1	14	2	−2	26	2	−1	Doubtful at 170 mm.
1	−1	Not readable	2	−1	*6·5*	2	None	Not readable
1	None	*41*	2	None	7·5			
1	+1	50	2	+1	37			
0·75	−1	Not readable	1	−2	Not readable			
0·75	None	*72*	1	−1	36			
0·75	+1	Not readable	1	None	*23*			
0·5	None	Not readable	1	+1	Not readable			
			0·75	−2	Not readable			
			0·75	−1	101			
			0·75	None	*31*			
			0·5	None	Not readable			

The numbers in italics indicate the best result with that size of type. "Not readable" means as in Table I.

The size of the retinal image would be slightly decreased by the use of a concave lens, but the diminution in size is only 2 per cent., therefore the types are left at their nominal values in the tables. Actually the values would work out as 8·8, 5·9, 1·97, 0·98, 0·74 and 0·49 instead of 9, 6, 2, 1, 0·75 and 0·5 respectively.

The difference in acuity might be ascribed to insufficient brightness of the "blue" light, and this suggestion must be carefully discussed, because there is no satisfactory way of comparing the brightness of two lights of different colours. At the threshold both lights are physiologically equal, but equal multiples of the threshold value will not appear of equal brightness. Moreover, the result depends on the part of the retina on which the comparison is made.

At the commencement of each series the aperture of the sector was closed to measure the minimal opening at which the light is just visible by direct vision, the patch of light being larger than the rod-free area. The "red" light became invisible at readings of 1, 2 or more millimetres, whilst the "green" and "blue" were still visible when the shutter was supposed to be closed (this must be equivalent to less than 1 mm. opening). Thus judged by the threshold the "red" light was weaker than the "green" or "blue." Therefore, for any reading of the sector the "blue" was a higher multiple of the threshold than was the "red."

According to the Purkinje phenomenon the brightness of the "blue" does not increase so rapidly as that of the "red," therefore it may still be claimed that the failure to read the smaller letters was due to insufficient brightness. This well-known effect, that there is a different law relating intensity and brightness, suggests that there may be a different type of receptor for the long and short wave-lengths of the visible spectrum.

The effect of slightly eccentric vision was tested, and it was found that with "red" light central vision was best, but that with the larger type the letters were read with less intensity of "blue" when they were observed slightly eccentrically, this effect being related to the greater sensitivity of the peripheral retina to light from the short wave-length end of the spectrum (see p. 278). With "green" there was also a tendency for the larger type to be read with a lower intensity peripherally than centrally.

With red and green screens it seemed as if the 6-metre type was visible at almost the same intensity as the 9-metre type, whilst a marked increase in intensity was necessary to read the 2-metre type. This is probably due to the two former readings being on the steep part of the curve, just before the point of inflection where the curve commences to flatten out (see fig. 2). It is not due to eye movements, as an instantaneous exposure by a camera shutter did not diminish the acuity for the larger letters.

The question of dark adaptation must also be considered. It is obvious that with the higher intensities the eye must have been adapted to fairly light conditions. With the lower intensities dark adaptation was present

Table III.

July 15, 1929. J.L.B. No artificial pupil.

Screen 8 (red).		Screen 10 (green).			Screen 12 (blue).		
Type.	Sector.	Type.	Sector.		Type.	Sector.	
	Central.		Central.	Eccentric.		Central.	Eccentric.
m.	mm.	m.	mm.	mm.	m.	mm.	mm.
8	16	8	4	1	8	18	2
3	36	3	17	15	3	127	110
1·6	34	1·6	38	31	1·6	Not readable	Not readable
1·1	109	1·1	63	61			
0·75	Not readable	0·75	Not readable	Not readable			

(In this experiment transparent letters on a dark background were used.)

December 17, 1929. H.E.R. No artificial pupil.

Screen 8 (red).				Screen 10 (green).				Screen 12 (blue).			
Type.	Lens.	Central.	Peripheral.	Type.	Lens.	Central.	Peripheral.	Type.	Lens.	Central.	Peripheral.
m.		mm.	mm.	m.		mm.	mm.	m.		mm.	mm.
9	None	5	8	9	None	4	2	9	−1	17	9
6	None	4	9	6	None	3	3	6	−1	45	25
2	None	9	Not readable	2	None	7	11	2	−1	Not readable	Not readable
				1	None	22	Not readable				

and fairly well marked. If dark adaptation were not complete it would not make much difference to the threshold readings in "red" light, but greater dark adaptation with "blue" would make the threshold lower and therefore exaggerate the difference between the threshold and the intensities at which the different types became visible.

In order to make clear the differences between the visual acuity in the light from the various screens, the average results of two experiments are shown in fig. 2, where the interrupted lines indicate the probable course of the acuity/intensity curves. The 0·5 m. type was read at 13 mm. with "whole" light, so the sector was fully opened to 170 mm. and distance increased by moving the head away until the type was just legible. As the distance was about 75 cms. the approximate acuity was 1·5.

The arrows in the diagram at 170 mm. for "red," "green," and "blue"

show that 0·5 m. and 2 m. types respectively were not read at 170 mm., therefore visual acuity at 170 mm. would be less than 1·08 and 0·27 respectively, and the curves should run slightly below the interrupted lines. From

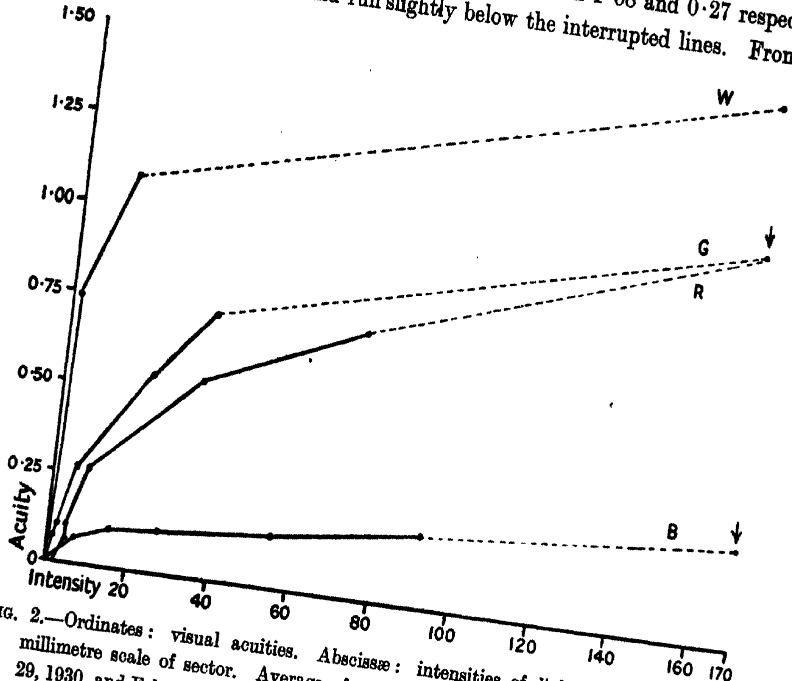


FIG. 2.—Ordinates: visual acuities. Abscissæ: intensities of light as measured on millimetre scale of sector. Average of results of experiments on H.E.R. (January 29, 1930, and February 5, 1930). W, whole light from lamp. G, light through screen 10 (green). R, light through screen 8 (red). B, light through screen 12 (blue).

positions of interrupted lines it is seen how it happens that occasional individuals will read 0·5 m. type with "green" light while others may read 2 m. type with "blue."

With screens 8 (red) and 10 (green) the acuity is comparable, showing a great increase for small differences in intensity when the intensity is low, but with higher intensities the curves show a sharp bend, indicating that the limit of visual acuity is almost attained and that much higher intensities would not show much better acuity. The green filter allows a higher brightness to be reached, hence that curve is slightly above the one for red. The entire mixed white light is much brighter than the small fractions of it which pass through the filters, so the curve for it is at a higher level.

With screen 12 (blue) the lower portion crosses the curve for screen 8 and the rise of the lower part of the curve is less steep than for the other two screens.

This probably corresponds to the falling off in the ratio of brightness to intensity with "blue" light contrasted with "red." It is doubtful, however, whether the acuity would ever reach as high a value as with the other filters.

There is an experimental limit to the increase in brightness of coloured lights. The intensity of light to be obtained by the use of a spectroscope is limited, and when a monochromatic patch is spread over a sufficient area to read letters the illumination is low. With colour filters large areas of moderately bright light can be obtained, but with increase in intensity of the light source wider bands of the spectrum will be raised to threshold value. In fact, whatever analytical mechanism exists in the retina it is possible that sufficiently high intensities of monochromatic light may stimulate all receptors, hence the approach to white of all colours at high intensities.

Therefore with increasing intensity the curves might continue to rise to the maximum value for white light, but the flattening of the curves can be taken to indicate the approach to the limit for light of the "colour" for the corresponding screen. In view of the fact that with screens 10 and 12 superimposed, light was visible at higher intensities, part of the visual acuity with screen 12 may be due to "green" light, and it would continue to rise to higher values if the source of light were indefinitely increased.

An attempt was made to use narrow bands of light from a spectroscope but (owing to "stray" light) the bands were not pure enough. The slit through which the narrow band came was treated therefore as the slit of a second spectroscope, and by means of a prism and lens it was focussed on another slit-like opening. This served as a means of illuminating the ground-glass of the apparatus shown in fig. 1, and records were made of the size of type which was readable. The intensity was reduced by means of a photometric wedge until the light became invisible. Thus the intensities could be expressed as multiples of the threshold (Table IV).

The interesting region is the blue green, in which it is seen that the visual acuity was decidedly higher than in the blue, but the multiple of the threshold is about the same in both, thus showing the influence of longer wave-lengths on the improved visual acuity and the need of a restricted "blue" light if the poor visual acuity with short wave-lengths is to be demonstrated. Earlier experiments with a less selective screen (11) did not show such marked results as those with screen 12. With white light the acuity is greater than unity (probably about 1·5), with screens 8 and 10 it lies between 2/3 and 1, whilst with screen 12 it is usually below 1/4.

Table IV.

Range of wave-lengths in Å.	Test type.	Approximate multiple of threshold.	Visua acuity.
6828–6752 (red)	0·75 m. readable 0·5 m. not readable	6918	between 0·72 and 1·08
6503–6304 (red)	0·75 m. readable 0·5 m. not readable	19608	between 0·72 and 1·08
5852–5701 (yellow)	0·75 m. readable 0·5 m. barely readable	22222	almost 1·08
5352–5250 (green)	0·75 m. readable 0·5 m. not quite readable	83333	almost 1·08
4893–4819 (blue green) —1 D lens used.	1 m. readable 0·75 m. barely readable	10753	almost 0·72
4669–4623 (blue)— 1 D lens used.	2 m. type readable 1 m. type not readable	19608	between 0·27 and 0·54

König found equal acuities for "white," "red" and "green" lights; but when one compares his maximum intensities (of the original light before passing through the filters) it seems probable that the stimulating light must have been anything but pure. The "red" light seems the most efficient and approaches the "white" in value. Thus, comparing his intensities when a given visual acuity was reached, we find :—

Visual acuity about	"White" light.	"Red" light.	"Green" light.
1·0	13·6	69·4	2269
1·5	119	2623	88590

"Blue" light—Maximum acuity 0·640 with intensity of 629800.

Either very little light passed through his "blue" filter or the short wave-lengths of light are very inefficient for form recognition.

Comparing the results with screens 8 and 12 the ratios of wave-lengths given on p. 278 indicate that the resolving power should be better with the latter in the ratio of 2 to 3, but they are inferior in the ratio of about 3 to 1. The only optical explanation for this difference is that greater scattering of the shorter wave-lengths occurs in the media of the eye, so that the dark parts of the image are illuminated by the scattered light, thus causing blurring of the

images. If much scattering of light occurred there should be a more marked halo round the illuminated field on which the letters are shown. With the higher intensities there is always a sensation of slight " haze " surrounding the illuminated field, but this was not noticeably greater with screen 12 than with the others.

An attempt to determine the effect of scattering in so far as the vitreous humour is concerned was made as follows :—by means of a lens an image was formed so that the letters could be photographed ; close to and in front of the photographic plate was placed a trough 5 cms. in thickness ; vitreous humours from bullock's eyes were filtered through cloth and centrifugalised to remove the pigment which became mixed with them ; the resulting solution was placed in the trough and photographs were made on panchromatic plates.

There was not much to choose between the definition of the photographs, which was the more remarkable as the letters were not clear enough to focus with the " blue " screen, and the photographs were obtained by focussing for white light and then making a series of exposures, moving the lens slightly closer to the plate before each exposure until the definition reached a maximum and began to decrease again. With " red " light the letters could be focussed, but for comparison a series of photographs was made as with " blue " in order to be certain of obtaining the best definition.

If failure to read the type in " blue " light is not due to scattering, it must be due to some physiological condition in the retina. Just as fineness of detail in a photogravure depends on the size of mesh of the screen, so the visual acuity will depend on the size of the functional areas of the retina. In other words, the smaller the area of retina corresponding to individual fibres of the optic nerve the greater the possibility of distinguishing detail.

Histologically it has been shown that groups of rods are linked to a single ganglion cell, whilst the number of cones linked to a ganglion cell is much less ; therefore if " blue " light stimulated rods, whilst longer wave-lengths stimulated cones in addition, one could account for the poorer acuity with screen 12. Against this explanation is the statement that there is a rod-free area in the retina, and this would have to be blue blind. It is true that the sensitivity to shorter wave-lengths is less in the fovea, yet they do stimulate that region. Parsons, in discussing the rod-free area, points out that the structures in the fovea are not conical ((4), p. 215, and (5), p. 236). He states : " This change in structure of the cones may be evidence of some physiological combination of the functions of both rods and cones in this situation " ((4), p. 12).

Certain experimental results can be interpreted on the working hypothesis

that colour vision in man behaves as if it were due to colour filters in front of some of the receptors (7). If there were red filters, yellow filters, and unscreened receptors, the unscreened receptors would be the only ones to be stimulated by light of short wave-lengths, whilst longer wave lengths would stimulate in addition receptors with coloured screens. Therefore "blue" light would stimulate fewer receptors per unit area, and visual acuity would be lower than for light from other regions of the spectrum. These two explanations of the poorer form vision with screen 12 are not mutually exclusive, as the unscreened receptors outside the rod-free area may be the rods.

That the rods may be especially related to vision of the short wave-length end of the spectrum is a view that has been expressed by others (3 and 9). Furthermore, if the interpretation of the results is that blue light stimulates rods and not cones, we should expect confirmation by other observations. Pieron (6) has shown that for peripheral vision there is a different relation between the duration of exposure and intensity of illumination for "blue" and for "red."

The phenomenon of so-called secondary excitation of the retina is possibly due to stimulation of rods by light of all wave-lengths. When a fairly bright spectral "red" or "green" light is viewed slightly eccentrically a bluish pair of arcs or an oval is visible. This is discussed elsewhere (8), and the explanation is suggested that the blue sensation is due to stimulation of rods by the halo of long and medium wave-lengths of light, which in the central region stimulate cones, giving rise to red and green sensations respectively.

Further, when the function of the rods is depressed or absent the threshold for blue should be raised. The colour vision in night-blind cases does not seem to have been thoroughly examined; Parsons states (4, p. 222): "Colour vision is normal with the exception of occasional diminution of sensitivity for blue lights."

Through the kindness of Mr. T. C. Summers, F.R.C.S., I was able to examine one case of retinitis pigmentosa. I found a diminished sensitivity to light, especially at the short wave-length end of the visible spectrum. This result, and the quotation from Parsons, is what one would expect when dark adaptation is defective; but on testing with wools in good light the patient confused blue and green, a mistake which is never made by the usual type of hypochromat. It is difficult to examine even intelligent individuals, because their visual fields are restricted and their form vision may not be good; however, there was no doubt that this case was defective in the discrimination of blue. König found that such cases were blue blind ((2) p. 718), and Traquair states (10) that

in cases showing interference with the retinal outer layers, with decrease in intensity of light, there is a reduction in the recognition of blue.

A further consideration to be borne in mind is that the relation of visual acuity to colour is not proportional to wave-lengths, because the results with red (6800 Å.) and green (5300 Å.) are comparable, but both are quite different from those with blue (4500 Å.). This sudden break is clear evidence that unless there is some critical relation between the wave-length and scattering in the eye media, the effect observed is not due to physical differences, but probably indicates a physiological difference, *e.g.*, in the type of receptor.

Summary and Conclusions.

1. Visual acuity has been tested with different intensities of light, using filters which allow only restricted regions of the spectrum to pass through.

2. Definition with short wave-lengths is less accurate than with medium or long wave-lengths.

3. The results are discussed with reference to the relative intensities, chromatic aberrations and scatterings of the lights used. That such physical factors can be the whole cause of the observed poorness of visual acuity under illumination with light of short wave-lengths seems improbable. More likely appears the involvement of some purely physiological factor, such as the existence of a lesser density of the specific receptors per unit of retinal area.

4. The lesser density of specific receptors per unit area may be due to colour filters in front of certain receptors, so that " blue " can stimulate only some of them, whilst longer wave-lengths such as "green" and "red" can stimulate a larger number per unit area. On the other hand, the difference might be due to a number of rods being linked to a single ganglion cell, whilst there may be a separate ganglion cell for each cone; therefore the areas served by single ganglion cells would be greater for impulses coming from rods than from cones. Supplementary evidence is given to support the view that the receptors limited to the reception of short wave-lengths stimuli are the rods.

I have much pleasure in thanking the Trustees of the London Hospital Research Fund for a grant from which the expenses of this research have been defrayed. I also have much pleasure in acknowledging the interest and constructive criticism of Sir Charles Sherrington during the writing of this paper.

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Further Observations on the Ultra-Violet Absorption Spectra of the Serum Proteins.—The Specific Extinction Coefficient of Serum Pseudo-Globulin.

By F. Campbell Smith (Freedom Research Fellow) and J. R. Marrack.

(Communicated by C. L. Evans, F.R.S.—Received March 8, 1930.)

(From the Hale Clinical Laboratory, the London Hospital.)

In a recent paper (Smith, 1929) one of the present writers described the ultra-violet absorption spectra of the serum proteins. The object of this work was to establish absorption curves which could be taken as reliable and which could be used as a standard of reference in later investigations. The writers (Marrack and Smith, 1930), working on the composition of diphtheria toxin-antitoxin floccules, have recently shown that these floccules are composed mainly of pseudo-globulin. In this investigation the writers' conclusions as to the nature of the floccules were based mainly on their ultra-violet absorption curves. The value of 1·2 was used as the extinction coefficient at the head of the curve for a 0·1 per cent. solution of pseudo-globulin of 1 cm. thickness (Smith, 1929).

In 1928, a paper by Svedberg and Sjögren appeared, on the molecular weights of serum albumin and globulin. It was there suggested that during the preparation of the serum proteins certain changes might occur in their structure,

resulting in the formation of substances composed of smaller molecules; this, they asserted, would be more liable to occur when elaborate methods of preparation were used. The figure obtained by Svedberg and Sjögren for the specific extinction coefficient of serum albumin is in close agreement with that found by the present writers. On the other hand, Svedberg and Sjögren obtained a figure of 22 for the specific extinction coefficient of serum globulin, as compared with the writers' figure of 12. It is manifestly important that the reason for the discrepancy should be ascertained.

The object of this investigation has been to repeat and amplify the writers' previous work. The extinction coefficients of a series of globulins, prepared both by the writers' and by Svedberg and Sjögren's method, have been measured.* Further, it has been shown that pseudo-globulin obeys Beer's law, and that therefore its extinction coefficient is specific.

Method.

The extinction coefficients of the series of globulins were measured by two independent methods:—

1. The Hilger quarter-plate spectrophotometer and rotating sector; the source of radiation being a condensed spark between tungsten steel electrodes.

2. By means of a sodium photo-electric cell in conjunction with a Hilger quartz monochromator. The circuit employed is shown in fig. 1.

In this circuit there is no leak between the electrometer needle and earth: hence a time exposure of 30 seconds is possible, with consequent increase of sensitivity. This is of importance, as a fine slit can be used to give sufficient purity of the part of the spectrum selected: if the slit is too wide a lower value is obtained than the real one and a serious source of error introduced. The source of radiation was a quartz mercury-vapour lamp. The extinction coefficient was measured at a wave-length of Å. 2804.

The potential produced by an exposure of 30 seconds through distilled water was compared with that produced by an exposure through the globulin solution. The potential produced is directly proportional to the amount of light falling on the cathode of the photo-electric cell. Thus, if V_1, V_2 be the potentials produced through distilled water and the solution, the extinction coefficient

* The specific extinction coefficient of (E) is given by the formula

$$E = \frac{\log I - \log I_1}{cd},$$

where I = intensity of light entering the solution, I_1 = intensity of light leaving the solution, c = concentration in grams per cent., d = thickness of layer, in centimetres.

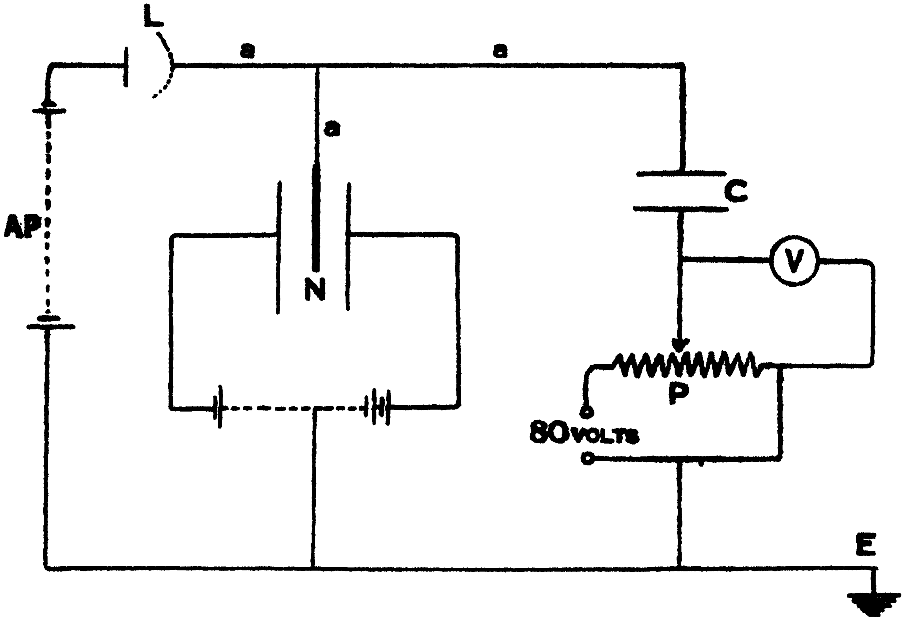

FIG. 1.

L. Photo-electric cell. N. Electrometer needle. C. Condenser. V. Voltmeter. AP. Accelerating potential (2–100 volts). P. Compensating potentiometer. *aaa*. Part of circuit not earthed. It must be highly insulated and well shielded. During the exposure of 30′ the electrometer needle N is kept at its zero by adjustment of P. At end of exposure, reading V is recorded, and exposure timed by shutter operated electrically by stop-clock. E is a connection to earth.

will be $\log V_1/\log V_2$. The cells employed were 0·5 cm. in thickness. The concentration of the globulin solution was of the order of 0·1 per cent.; the actual preparation and dialysis was carried out in the cold, at a temperature of 1° C.

Experimental.

The experiments performed may be classified under the following heads :—

1. Comparison of the extinction coefficients of serum globulin, as prepared by Svedberg and Sjögren's method, and by that of the writers.

2. Measurement of the extinction coefficient of normal sera.

3. To ascertain whether pseudo-globulin obeys Beer's law.

1. The essential difference between Svedberg and Sjögren's and the writers' method of preparing serum globulin is as follows :—Svedberg and Sjögren, after precipitating the globulins by half-saturation with ammonium sulphate, dissolved the precipitate in 10 per cent. saline or phosphate buffer solution and

dialysed against a phosphate buffer of a *p*H of 5·5, toluene being used as a preservative. In this way the separation into pseudo-globulin and eu-globulin was prevented; the resulting product, in their opinion, was serum globulin. In the writers' method of preparation the eu-globulin fraction was thrown out of solution, both by dialysis against distilled water and by one-third saturation with ammonium sulphate; in unpublished experiments the writers have shown that the absorption of eu-globulin is identical with that of pseudo-globulin. The resulting pseudo-globulin solution was used for the absorption measurements.

A series of globulins was prepared by the above two methods. The extinction coefficients obtained are shown in Table I. The total nitrogen was measured by Kjeldahl's method; the specific extinction coefficient was calculated from the absorption measurements and the total nitrogen.

Table I.

Sample No.	Percentage of protein, by Kjeldahl's method.	Specific extinction coefficient.		Method of preparation.*	Remarks.
		Photographic method.	Photoelectric cell method.		
1	0·097	11·4	—	S. and M.	At *p*H of 7.
2	0·080	12·5	—	S. and M.	At *p*H of 3.
3	0·110	12·7	12·2	S. and M.	
4	0·160	12·0	—	S. and M.	Diphtheria antitoxin, heated for 1 hour at 60° C.
5	0·109	11·9	—	S. and M.	Stood for 4 days, at *p*H of 3·4.
6	0·045	—	12·0	S. and M.	At *p*H of 5·5.
7	0·045	—	12·0	S. and M.	At *p*H of 8·5.
8	0·083	12·0	12·0	S. and M.	Diphtheria antitoxin.
9	0·100	12·0	—	S. and M.	
10	0·110	12·0	—	Sv.	Dialysed in cold, against a buffer solution at *p*H of 5·5.
11	0·109	11·9	—	Sv.	At *p*H of 7, without toluol as preservative.
	0·109	11·9	—	Sv.	At *p*H of 3, without toluol as preservative.
	0·104	12·0	—	Sv.	At *p*H of 7, with toluol as preservative.
	0·104	12·0	—	Sv.	At *p*H of 3, with toluol as preservative.
12	0·103	—	12·1	Sv.	
13	0·101	11·1	11·1	Sv.	

* S. and M. = authors' method of preparation; Sv. = Svedberg and Sjögren's method.

2. Svedberg and Sjögren have suggested that the composition of serum globulin may be altered during its preparation; it has occurred to the writers

to investigate the extinction coefficient of fresh normal serum, for which a minimal amount of preparation is required.

Three samples of horse serum were taken, two being normal and one containing diphtheria antitoxin. The ratio of albumin to globulin was estimated gravimetrically. The serum was diluted five times with a 1 per cent. saline solution, this was then added to its own volume of saturated ammonium sulphate. The globulin was filtered off and the albumin estimated in an aliquot part of the filtrate. The total protein nitrogen was measured by Kjeldahl's method. The serum was diluted with 0·9 per cent. NaCl, to give a concentration suitable for spectrophotometric analysis, and its extinction coefficient measured. This was compared with that calculated from the ratio of albumin to globulin, using a specific extinction of 12 (the writers' figure) and 22 (Svedberg's and Sjögren's figure) for globulin and 5·8 for albumin.

Table II.

Column I = albumin/globulin ratio.

Column II = total percentage of protein. estimated by Kjeldahl's method.

Column III = extinction coefficient. calculated from columns I and II, taking specific extinction coefficients of albumin and globulin as 5·8 and 12 respectively.

Column IV = extinction coefficient, calculated from Svedberg and Sjögren's figures of 5·8 and 22, for the specific extinction coefficients of albumin and globulin.

Column V = extinction coefficients, found experimentally.

Sample No.	I.	II.	III.	IV.	V.
1	3/4	7·0	65·4	105·4	70
2	3/4·5	7·5	71·4	116·4	80
3	3·5/5	8·5	80·3	130·3	90

3. In order to ascertain whether pseudo-globulin obeys Beer's law, the following two methods of investigation were adopted:—

A.—By measuring the extinction coefficient, keeping the product of the concentration of the solution (c) and of the thickness of the layer (d) constant. The thickness was varied by a specially constructed micrometer cell. As can be seen from Table III, the extinction coefficient is the same for the different values of c and d employed.

Table III.—Normal Serum Globulin, prepared by Svedberg and Sjögren's Method.

Concentration per cent.	Density of layer, in mm.	Extinction coefficient ± 0·04.
0·1	10·0	1·2
0·2	5·0	1·2
0·4	2·5	1·2
1·0	1·0	1·2
2·0	0·5	1·2

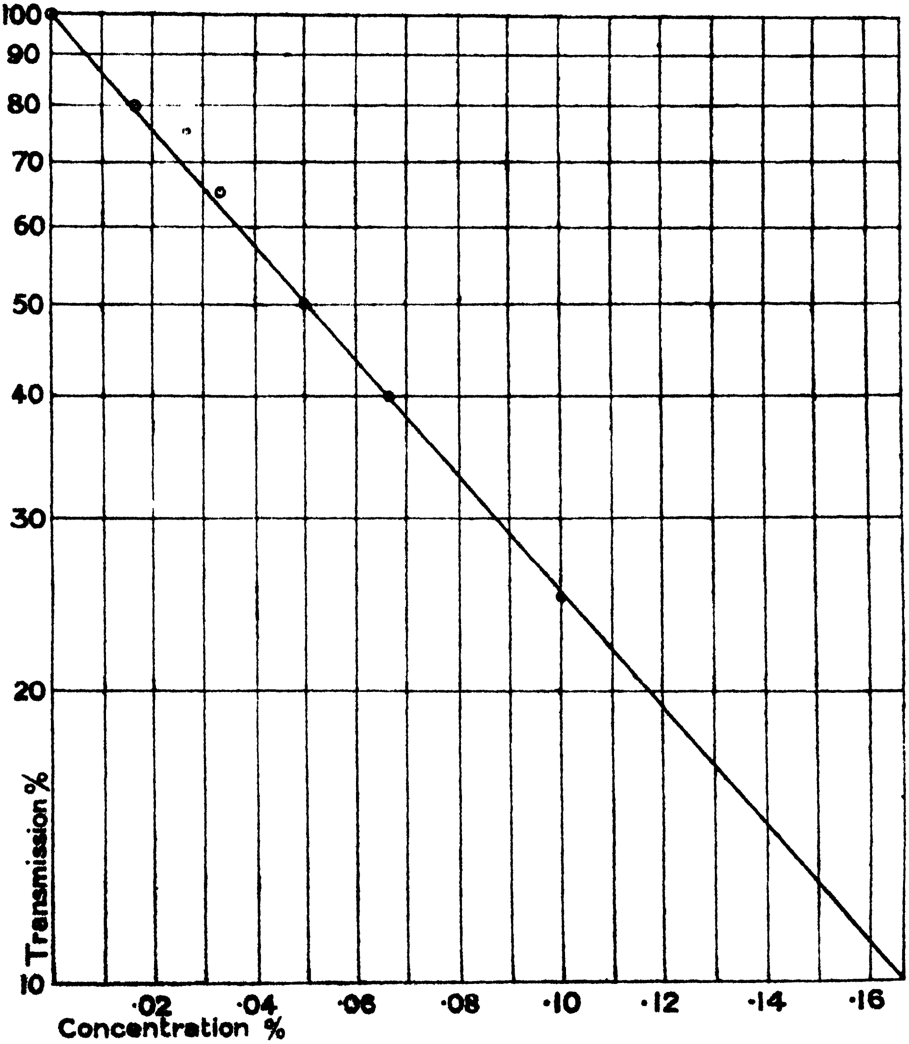

Fig. 2.

B.—By measuring by the photo-electric method, the extinction coefficients of a series of dilutions of pseudo-globulin. A straight-line relationship is seen to exist between the extinction coefficient and the concentration, the thickness being kept constant at 5 mm. (fig. 2).

From the evidence given above, the writers feel that they can confirm the figure previously given by one of them, namely 12, for the specific extinction coefficient of serum globulin. They are unable to suggest any source of experimental error that might explain the discrepancy between this figure and that obtained by Svedberg and Sjögren, especially in view of the fact that the extinction coefficient has been confirmed by an independent photo-electric method.

They would especially call attention to the results obtained in the case of blood serum; this would seem to offer conclusive evidence in favour of their own results. They can find no evidence that would suggest any alteration in the composition of serum globulin during its preparation, as has been suggested by Svedberg and Sjögren.

Summary.

1. The value for the specific extinction coefficient of serum pseudo-globulin, as given by one of the writers (F. C. S.) in a previous communication, has been confirmed.

2. The method of preparing and separating serum globulins does not appear to affect their ultra-violet absorption spectra.

3. The extinction coefficient has been measured by a photographic and by a photo-electric method.

4. Serum pseudo-globulin has been found to obey Beer's law.

The authors are especially indebted to Messrs. Adam Hilger & Co., Ltd., for the loan of a quartz monochromatic illuminator and to the Staff of the General Electric Company, Ltd., for valuable advice concerning their photo-electric apparatus.

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Discussion on Geological Climates.

The Discussion was opened by Dr. G. C. Simpson, F.R.S., and the following took part:—Prof. A. C. Seward, F.R.S., Prof. J. W. Gregory, F.R.S., Sir Peter Mitchell, F.R.S., Mr. C. E. P. Brooks, Dr. C. Tate Regan, F.R.S., and Dr. Hamshaw Thomas.

(Received February 12, 1930.)

Dr. G. C. SIMPSON, F.R.S.: It appears to me appropriate that a meteorologist should open this discussion, for the study of climates is a branch of meteorology. But while I speak as a meteorologist, I cannot speak for meteorologists, because on this subject there is no formulated meteorological opinion. What I say, therefore, must be taken as my personal opinion, with which other meteorologists may or may not agree.

During the last few years I have given much thought to this problem of past climates and I have come to certain definite conclusions. I wish to lay these conclusions before you this afternoon, but in the short time available I can do little more than state them; I cannot hope to defend them by a reasoned exposition, but in most cases that has already been given elsewhere.*

The earth is a sphere and is in rapid rotation about an axis, which throughout geological time has maintained an inclination of approximately $66\frac{1}{2}°$ to the plane of the ecliptic. These two facts combined lead me to my first conclusions:

(*a*) There must always have been climatic zones with the equatorial zones warmer than the polar zones; in other words, the mean temperature of the zones must always have decreased from the equator to the poles.

(*b*) There must always have been a summer half-year and a winter half-year.

The actual climate within a zone depends on two factors: (1) the intensity of solar radiation and (2) the distribution of land and water.

A study of the existing climatic zones leads to the following conclusions regarding the effect of the distribution of land and water:—

(*c*) The mean temperaure of a climatic zone is almost entirely unaffected by the distribution of land and water; and as a corollary to this the mean temperature of corresponding zones is always the same in both hemispheres.

* G. C. Simpson, "Past Climates," 'Quart. Jour. Roy. Met. Soc.,' vol. 53, p. 213 (1927); "Further Studies in Terrestrial Radiation," 'Memoirs Roy. Met. Soc.,' vol. 3, No. 21 (1928); "Past Climates" (The Alexander Pedler Lecture, 1929), 'Mem. Manchester Lit. & Phil. Soc.,' vol. 74, p. 1 (1929).

(*d*) The presence of land and water within a zone leads to the mean annual temperature departing locally from the mean of the zone; but these departures seldom exceed 5° C.; any larger departures are not only rare but limited to very small area—for example, in the neighbourhood of a warm or cold oceanic current.

(*e*) The distribution of land and sea within a zone affects chiefly the annual range of temperature and the distribution of rainfall.

The bearing of these last conclusions on the problem of past climates may be expressed:—

(*f*) No change in the distribution of land and sea alone could have produced the large changes of climate shown in the geological record.

Turning now to the second possible cause of variation—a change in the intensity of solar radiation—we come to a much more difficult problem for we have little or no observational data to go on, and the theory, which involves the equilibrium of incoming and outgoing radiation, is extremely difficult. Still I think we can arrive at some useful conclusions.

We have already seen that owing to the shape of the earth there must always be a fall of temperature from the equator to the pole. Now it is not difficult to see that this difference of temperature must increase as the solar radiation increases. The difference must obviously be nil when the radiation is nil, while with our present radiation it is 50° C., and as every increase in solar radiation adds more energy to equatorial than to polar regions it is difficult to see how any other effect could result. The matter, however, is not quite so simple as this, but as the result of a more detailed study I feel justified in stating:

(*g*) An increase in solar radiation leads to an increase in the temperature difference between the climatic zones.

As the general circulation of the atmosphere depends on the difference of temperature between the climatic zones a corollary to (*g*) is

(*h*) An increase in solar radiation leads to an increase of the general circulation of the atmosphere,

and as the amount of cloud and precipitation depends on the general circulation of the atmosphere, a corollary to (*h*) is

(*i*) An increase in solar radiation leads to an increase in cloud and precipitation.

But an increase of cloud has a great influence on the incoming radiation. Cloud reflects something like 78 per cent. of the incoming solar radiation and the energy thus reflected takes no part in the heat balance of the earth and its atmosphere. Thus an effect of increasing the solar radiation is to decrease the proportion of the solar radiation which is available for warming up the earth, and there is good reason to believe that it is this increase in cloud amount which is the main factor in effecting a new balance between the incoming and outgoing radiation. On account of this effect of the cloud it appears likely that the actual change of temperature at the surface is not very large; but it is easy to show that an increase of solar radiation must lead to an increase of temperature in all zones.

Let us consider the present polar regions. In the course of the year each polar region radiates much more energy than it receives directly from the sun. This expenditure over income is made good by the heat transferred from lower latitudes by the general circulation of the atmosphere. Now I have already shown that the increase of solar energy results in an increased general circulation, hence the polar regions will receive two additions to their heat supply when the solar radiation increases; first from the increased solar radiation itself,* and, secondly, from an increased supply from lower latitudes through the agency of the general circulation. Thus the temperature of the polar regions must increase. This combined with (*g*) gives us:

(*j*) An increase in solar radiation leads to an increase in the mean temperature of all zones.

Let us now examine some of the geological problems in the light of these conclusions, assuming that there has been no shift of the pole and no drift of the continents.

There appears to be satisfactory evidence that in the late Carboniferous period there was extensive glaciation in the southern hemisphere, while land ice extended right down to sea-level at many places in equatorial regions. At the same time Europe and high northern latitudes appear to have enjoyed much more temperate, if not sub-tropical, conditions. This is an obviously impossible arrangement of climatic zones, for the temperature of the zones in the northern hemisphere would increase from the equator towards higher latitudes, which is against our first conclusion; and further, there would be a

* As the reflecting power of snow is practically the same as that of clouds, changes of cloud amount in polar regions do not affect appreciably the effective solar radiation.

radical difference between the temperature of corresponding latitude zones in the two hemispheres, which is against conclusion (*c*).

Turning now to the conditions in the Eocene period, it is generally supposed that during this period the temperature in all parts of the world was remarkably uniform, and that the temperature in high latitudes was much higher than at present. This is again an impossible result, for a temperature more uniform than the present could only have been due to a decrease in solar radiation (conclusion (*g*)), and this would have resulted in a general lowering of the temperature of all zones (conclusion (*j*)).

Finally, we are told that the Pleistocene Ice Age was due to a general lowering of the temperature over the whole globe, which could only have been due to a decrease in solar energy much below that at present. This would have resulted in a decrease in the intensity of the general circulation of the atmosphere, with decreased cloud and precipitation (conclusion (*h*)). But during the Pleistocene we had one or more pluvial periods affecting nearly all parts of the globe, which would only have been brought about by a greatly increased general circulation and much more precipitation.

In the few minutes which remain to me I will sketch out my own views on past climates. During the Pleistocene there were large changes in climate, the glacial and interglacial periods, which were unaccompanied by any appreciable changes of the physical state of the earth. These changes can only have been due to changes in solar radiation, and I have endeavoured to show elsewhere that there were probably two large oscillations of intensity of solar radiation during the Pleistocene, giving the four advances of the ice in the Alps and two pluvial periods in the lower latitudes.

In my opinion changes of this nature, limited in magnitude and of relatively short period (in the geological sense), have always occurred in all geological periods, due to periodic variations in the intensity of solar radiation.

The large climatic variations which have caused the climate of Europe to be temperate to-day, frigid in the Pleistocene, sub-tropical in the Miocene and tropical in the Carboniferous, and have given sub-tropical if not tropical conditions in polar regions and polar conditions in equatorial regions, can only be explained by Wegener's hypothesis of the shift of the poles and the drift of the continents, the climatological consequences of which have been worked out in such great detail in Köppen and Wegener's book, 'Die Klimate der geologischen Vorzeit.'

Prof. A. C. Seward, F.R.S.: I propose to deal, quite briefly, with some of what Dr. Simpson called, in one of his papers, "the hard facts of geology."

The question is, whether some of those " facts " are really facts, and whether some of us who are particularly interested in evidence afforded by plants accept some of the statements frequently found in text-books.

In the first place, I want to emphasise, speaking, of course, only for myself, the view that there is no justification for describing the climate of certain past periods in the Northern Hemisphere as tropical, on the evidence afforded by fossil plants. Dr. Simpson spoke of the climate in the Northern Hemisphere during the closing days of the Carboniferous period as tropical. I do not think there is any real evidence to support such a statement. Secondly, we have been told that the climate at certain periods was uniform. None of us, I think, really believes—we cannot believe—that the climate of the world was ever uniform. Special allusion was made to the early tertiary floras as examples of a uniform vegetation; there is clear evidence, when we compare floras from the far north, in Greenland, many localities in Northern Asia, and across Siberia, including also the tertiary floras of Mull and Northern Ireland, with the floras of the same geological age further south, we find many clearly marked differences. On the one hand there is a temperate and more northern type of vegetation; on the other a southern which we should now call sub-tropical. If I may be pardoned for a personal remark at this stage, about 40 years ago—it may be rather longer than that—I wrote a so-called essay on a set subject—" Fossil Plants as Tests of Climate." I hope that some of the things I stated then will not now be quoted as evidence against me.

There is no doubt that fossil floras are of very great value as guides to changes in climate; on the other hand, the value of these guides has, I think, been over-estimated. Let us consider, first, some of the older floras. The evidence of climate afforded by them is of little value, as the plants are all extinct. Some of the plants belong to extinct groups, and it seems impossible to attempt to draw conclusions as to climate by comparing plants which are extinct with plants remotely related to them which are alive at the present time. Further, when we come to the more recent geological floras, the later Cretaceous and the Tertiary, we must remember that the species are practically all different from any that are now living. We know at the present time what tremendous differences there often are in power of resistance to climatic conditions between species belonging to the same genus.

Again, many of the older floras consist, almost entirely, of plants belonging to the fern group and to the gymnosperms. In these older floras we do not find representatives of the present dominant class of plants in the world, namely, flowering plants. If we eliminated flowering plants from the

vegetation of the present day we should find resemblances between the different floras of the world would appear to be much greater than they actually are. The chief and most striking differences are given by flowering plants rather than by plants lower in the scale of plant-life. Let me give examples to support the points I am trying to make.

First, the present distribution over the world of different families and genera of plants cannot be taken as an accurate measure of their resistance to external factors. Think of the conifers included in the genus *Araucaria*, which we are familiar with in our parks and gardens. Why is it that that genus exists only in certain parts of the Southern Hemisphere, where it has a discontinuous distribution, and is absent from the Northern Hemisphere? Take the genus *Sequoia*, the mammoth tree and redwood of California, which is restricted to a narrow border on the Pacific Coast; why do not these plants exist in other parts of the world, where the climate would appear to be just as favourable for their existence as in the places where they still survive?

This brings me to a suggestion—I cannot prove the correctness of what I am going to say—in the course of ages, plants may have, and probably have, altered in their constitution. What I mean is this: we know that a certain plant refuses to grow in a particular climate; it requires a warmer one: is there any good reason to believe that ancestors of the plant may not have been more hardy or more tolerant to external conditions than are the living forms? I do not think it is an unreasonable suggestion to offer that in the course of time—a few millions of years—races of plants may have passed from a stage of youth and vigour and adaptability to external influences to a condition of comparative senility. At the present time we find that certain plants have a very restricted area of distribution; on the other hand, when we look at the records in the rocks, we discover that species closely related to those which still exist were almost cosmopolitan. I think one explanation of this contrast may be an alteration in the power of resistance of plants in the course of their long history.

Dr. Simpson made a very clear statement on the possible effect on climate of alterations in the distribution of land and sea. Of course, one is bound to accept—and I do accept, willingly—the fundamental facts which Mr. Simpson has brought before us, but I cannot help thinking that he has rather underestimated differences that might be made within a certain zone by altering the distribution of land and sea.

[*Slide.*]

We are concerned not only with mean temperature of a whole zone, we are interested also in the variations in climate within a zone. In this map [*slide*] of the Arctic region, a line is drawn showing more or less accurately the tree limit, that is to say, the northern boundary of trees reaching a height of, say, 15 feet or more. Close to Cape Farewell a few trees reach a height of 10 or 12 feet. When we pass to latitude 69° N. and latitude 70° N. the dwarf willows are not more than 3 feet in height. In Alaska the white spruce reaches 50 or 60 feet. The difference between the two kinds of vegetation is very considerable. (The black dots on the map I have not time to deal with; they were put in, some time ago, to show the places where many of the fossil plants from Arctic regions have been collected.) Dr. Simpson spoke of the rich Carboniferous flora, which has been found in Spitsbergen. Attention may be called to the rich Upper Devonian flora of Ellesmere Land, to the Rhaetic flora of Scoresby Sound, also to several Arctic Jurassic floras, and to the rich Lower Cretaceous flora of Western Greenland. The plants found in Ellesmere Land, of late Devonian age, seem to differ in no respect, so far as we can see, from plants of the same age collected in South Russia and in Southern Ireland. Similarly, the Rhaetic flora of East Greenland is just as rich and composed of plants with as large stems and leaves as the flora of the same age found in the Southern part of Sweden.

Before I say anything about geological problems, I want to make a remark or two on the subject of uniformity. I know it is often stated—and I have made the statement myself—that the vegetation in past ages was more uniform than that of to-day. And that statement is, broadly I think, correct. Evidence in favour of greater uniformity in the world's vegetation is particularly striking when we compare Jurassic floras from different parts of the world; but, as I have said, this is in part at least due to the absence of all modern types of flowering plants. The tendency is to exaggerate the uniform nature of ancient floras. Moreover, if we examine the vegetation of the Carboniferous period, we find there were three or more well-marked botanical provinces: the vegetation was by no means uniform. Passing to the Triassic period, there is good evidence that the vegetation in the Northern Hemisphere differed from that in the Southern Hemisphere. Another reason why uniformity is often exaggerated is our imperfect knowledge of the extinct floras. The more we find out about the composition of these earlier floras, the more we realise that they show the same sort of zonation in vegetation as we have at the present time.

[*Slide showing a map of the World at the end of the Carboniferous Period.*]

The shaded areas in South America, Africa, including Madagascar, Australia and India areas, show approximately the regions where there is convincing evidence of widespread Palæozoic glaciation. In the Northern Hemisphere there is no such evidence on a large scale. An Upper Carboniferous or Lower Permian boulder clay has been described in the neighbourhood of Boston, in the United States, which may indicate the occurrence of local glaciers. The question is, was there so great a difference between the North and the South as Dr. Simpson suggests? I do not think there was. Dr. Simpson has described the vegetation of the Coal period in the Northern Hemisphere as tropical; a similar statement is common in geological books. It is impossible to find any satisfactory evidence in favour of such a conclusion.

Secondly, are we sure that, in spite of the presence of glaciers in the Southern Hemisphere, the climate over the great continent of Gondwanaland was as cold as Dr. Simpson believes?

[*A slide showing a photograph obtained through the kind offices of Dr. Cockayne.*]

The photograph shows in the background the Franz Joseph's glacier (44° S. lat.); in the foreground, tree ferns and a sub-tropical vegetation. Here, then, is a combination of glacial conditions and a sub-tropical flora. The combination is, I think, striking. It is conceivable that there may have been similar conditions in certain parts of Gondwanaland during the closing stages of the Paleozoic period.

[*Slide showing a restoration of the vegetation of Gondwanaland.*]

[*Slide of a restoration of Rhaetic vegetation in Eastern Greenland.*]

The reconstruction of a scene in Rhaetic Greenland is based on material collected by Mr. T. M. Harris, of Cambridge, in the neighbourhood of Scorseby Sound. Here one sees ferns allied to species now living in the Malay region, also plants closely related to the Maidenhair tree (*Ginkgo*). It does not follow from the presence in this Rhaetic flora of certain plants which have at the present time their nearest representatives in the Tropics and sub-Tropics, that the conditions were then sub-tropical. It is, in my opinion, probable that these extinct plants—which are ancient even in the geological sense—may have differed considerably from their present-day relatives in their reaction to external factors and in their ability to endure hardships.

Passing to the Jurassic period, the most striking example of a flora in what one feels is the wrong place, is afforded by the rich flora of Grahamland, on the edge of the Antarctic Circle. Some of the Grahamland fossils appear to

be specifically identical with plants obtained from Jurassic rocks in Yorkshire and in India.

[*Slide showing early Cretaceous vegetation of Western Greenland.*]

Here is an attempt to give a restoration of a scene in West Greenland in the early days of the Cretaceous period. With ferns, conifers and other gymnosperms are associated flowering plants, which are believed to be some of the earliest representatives of the present dominant class; not the oldest known flowering plants, but the earliest examples of modern types, such as plane trees, *Artocarpus* (Bread fruit), *Magnolia* and others.

[*Slide showing present northern limit of distribution of families and genera of plants represented in Cretaceous flora of Greenland.*]

A comparison of the present geographical range of the plants most closely allied to the Cretaceous genera with their former extended area of distribution would seem to indicate the prevalence in Greenland of a sub-tropical or even a tropical climate. But a conclusion which seems obvious is not necessarily sound.

Many flowering plants almost certainly originated in the Arctic regions, and gradually spread to the south. Spreading from the north to the south is not entirely a consequence of changing climate, though, no doubt, it is in part; migration is to some extent connected with competition with other and more recently evolved types of plants.

I should like to say, in conclusion, that I am grateful to Dr. Simpson, not only for what he has said to-day, but for what he has written in the last few years; it is only through the co-operation of meteorologists, geologists, and botanists that we can hope to arrive at a satisfactory solution of this very difficult and fascinating problem. And I would add that it is extremely difficult to explain some of the palæobotanical and geological facts unless we have recourse to Wegener's hypothesis.

Sir PETER CHALMERS MITCHELL, F.R.S.: I am not wholly able to comply with your invitation to start an opposition in this discussion here. I can only, in a few words, try to reinforce, from the point of view of terrestrial animals, the proposition of Prof. Seward. And I take it to be that it is extremely dangerous to infer from fossils the climate of the past, because of the present conditions under which you find kindred plants. It is true that, from the point of view of terrestrial animals, an extreme and prolonged period

of glaciation would naturally sweep off everything; it would destroy the land vegetation, and therefore the animals would have to disappear with the land vegetation. And it may be of interest for geologists to tell us when and where these changes took place so that we may infer their effect on geographical distribution; but of far more interest are the comparatively minor changes in climate, which Dr. Simpson attributed to alterations in solar radiation—changes which must have been comparatively small, and must have happened often. I am not sure that, in this respect, Dr. Simpson gave the information which is most useful to zoologists. He spoke of zones round the earth, zones being determined by the gradations of mean temperature. But land animals do not live by mean annual temperature; they live, chiefly, by their capacity to endure a certain range of temperature. You might have a room which was at a mean temperature between freezing point and the hot room of a Turkish bath. In that temperature I can imagine a man, a snake, a frog, and a lobster being very comfortable. But if they transport themselves into the open air at the lower temperature, the man might jump about happily and be quite comfortable—until arrested by the police—but the snake, the frog, and the lobster would be almost at once paralysed by the cold. And if these animals were transported to the hottest chamber of the Turkish bath, long before the man had begun to perspire, the other animals would be dead. The range of temperature is more important in determining the possibility of land animals to live than is the annual mean temperature. It is certain that the distribution of land and sea makes very important differences—much more than 5° of difference—in the range of temperature, as opposed to the annual mean.

With regard to present animals and range of temperature, there is one general observation we ought to make, and that is, that we are all accustomed to associate certain animals far too closely with a particular kind of temperature. We think of the higher apes, for instance, as being tropical animals. It is true that the gorilla lives in the low hot plains of West Africa, but it also lives and flourishes in places at elevations of 10,000 feet and upwards, where there are cold and frosty nights, and where the conditions are far from tropical. And, in the same way, the Japanese ape lives comfortably in districts which endure snow. For some years we have experimented in the Zoological Gardens on the range of temperature. We find that baboons live and breed in the open air, and if they have a cave to go into, they can come out in the snow and stand all the vagaries of the London climate. If you go through the list of living land animals you find that nearly all of them have a greater range of adaptability to temperature than we suppose. Cockatoos breed well in the open air,

without any warmth or shelter, in this country. Humming birds go up to the level of perpetual snow on Chimborazo ; and the little European tree-frog, which we associate with the Mediterranean shores, breeds in the Forest of Fontainebleau, where the temperature in the winter is very low, and the range of temperature is greater than here. I could multiply examples of this, which should be familiar to every zoologist, but they are often forgotten. And there is a good deal of evidence in support of a gradual evolution in the power of adaptation to a range of temperature. Even in cold-blooded invertebrates. like earth-worms, Vernon has shown that their output of oxygen did not go up and down definitely with the temperature, but that it varied independently of the temperature, as if they had some internal power of temperature regulation. We know that monotremes have a lower temperature and a lower power of adaptation and of control than have marsupials, while marsupials have a lower power than higher mammals. And I think there is every reason to suppose that there has been a gradual evolution of this power of adaptability to temperature. Take even creatures like fish. Dr. Tate Regan will correct me if I am wrong. Fresh-water fish are usually derivatives from salt-water fish, and fresh-water fish endure much greater ranges of temperature than do salt-water fish. In our own aquarium at the Zoo, in the tanks for salt-water creatures, we had to introduce a freezing system as well as a warming system, both being unnecessary for fresh-water fish. The change from salt water to fresh water has been accompanied by a greater power of adaptation to changes of temperature.

My general point is merely that we zoologists cannot infer from the presence of fossils any particular kind of climate. In fact, the inference must be reversed ; if there are fossils of a particular kind of animal found under conditions where. meteorologists and geologists tell us, there was such and such a climate, we know that these animals must have been able to endure those climates.

Prof. J. W. Gregory, F.R.S. : The main part of the problem for the geologist is the extent of the changes in climate which the meteorologist has to explain. There is great difference in the interpretation of the facts. Semper said, some time ago, that our notions of past climates are in the highest degree uncertain and confused. That uncertainty and confusion is due, in part, to the survival of various picturesque exaggerations, some of which I hope Dr. Simpson's contribution will do much to allay. Thus we were told that at various times the whole earth had a uniform climate. We now have Dr. Simpson's testimony that that was impossible.

Prof. Seward has called attention to variations in the tree line, which varies more than 20° in latitude on opposite sides of the Atlantic ; and the odds are against these thermal isanomalies being at their maximum at the present time. I think it is possible to explain all the geological facts by a reasonable increase in those isanomalies.

The evidence which has been mainly used as proof that tropical climates have occurred in the Arctic regions has been the distribution of coal seams and coral reefs. A lantern slide exhibited shows a seaside plain littered with tree trunks ; some with roots attached as if the tree had been blown over where it grew ; one trunk is erect. Between those logs are growing abundant plants, which, for their size, have rather large leaves. Let the plants accumulate until they fill up the spaces between the trunks ; cover it all with a sheet of sediment heavy enough, and there will be formed a coal seam. Some future observer may claim that it was formed under tropical conditions, but this photograph was taken in the Arctic region on the north side of Jan Mayen, 71° N., and I have seen exactly the same kind of thing 12° from the Pole, in Spitsbergen. These trees grew in Siberia, were carried down by the rivers, drifted across the Arctic Ocean and then stranded. This case shows that a coal seam may be formed in the Arctic under present conditions.

Dr. Simpson has referred especially to two periods : the Eocene and the Carboniferous. He has quoted the view in regard to the Eocene period that it had a world-wide uniform climate, and was hot at high latitudes. But geological evidence is against both views. The climate was not uniform, for while in Eocene times in the London basin it was sub-tropical, at the same latitude in British Columbia there were glaciers in existence ; and they occurred also in Colorado 10° further south. That the Eocene climate was not of a high temperature in high southern latitudes we know from the Antarctic. Wegener himself claimed glaciation at that time in Patagonia, and there is clear evidence of it in Grahamland. As to Northern latitudes, the evidence of the Eocene is meagre in the extreme. Except for a certain number of plant beds of dubious age, we know little about the Eocene in high northern latitudes. There is none in northern Europe ; in Asia the Eocene occurs in Turkestan but not in Northern Siberia ; and in North America the Eocene occurs to 50° N., but it is not tropical. In Greenland the one certain patch of Eocene, the Cape Dalton formation, has yielded a few shells and a bit of a beetle ; but no indications of tropical conditions. It may be said that if the conditions were not tropical in the Arctic in the Eocene period, they were so in the Cretaceous and Miocene. Prof. Seward has re-investigated the Cretaceous flora of Greenland

and has shown that it implies no change of climate which cannot be explained by alterations in the geographical factors. I support his testimony from the marine fauna. If the plant beds indicated tropical conditions in the Arctic a warm water fauna would have been expected ; but the marine fauna contemporary with the plant beds might have lived in the Arctic at present. If the Miocene land flora were tropical, why was the sea in the Behring Straits at the time occupied by a fauna that indicates colder conditions than those there at present ?

I refer to the coral reef argument as it has recently been re-asserted by Troedsson. He fortunately gives the dimensions of his corals, and his largest are crusts an inch thick or nodules only a few inches in diameter. Coral reefs should be made of much larger material. The small size of these corals indicates that they were growing under cold conditions ; and that climate was not tropical in the Arctic in Silurian times, the date in question, is shown by the glacial deposits in Alaska and British Columbia.

The Upper Carboniferous glaciation is the most difficult part of the problem with which the geologist has to deal. A map of the Gondwanaland glaciations shows that they occurred at about a dozen widely scattered centres on high land near the sea. Dr. Simpson says the ice occurred then in many places on the Equator at sea level. In most of the glaciated areas there are no indications of marine conditions. The glaciers reached the sea in South America at 35° S., but not in the tropical areas in Brazil ; the ice may have reached the sea in South Africa, south of 25° S. In India the evidence of marine intercalations appears clear for the Salt Range at 33° N., but further south in Central India, at 24° N., there is no evidence that the glacial deposits were laid down in the sea : the ice in that area came from the high Aravalli Mountains, which formed the great collecting ground. In Australia the most striking association of marine and glacial deposits was at 28° S. on the Irwin River ; like the rest it is outside the Tropics, and there a hot-water fauna—the goniatite (Gastrioceras), floated in shoals into this sea, and was destroyed in myriads by the cold. The monsoon which blew these shells south-eastward would have brought abundant moisture that would have been precipitated on the adjacent mountains, while a cold-water gulf from the Southern Ocean would have secured a heavy winter snowfall, which would not have been completely melted in the cold, wet, cloudy summer. Hence there was a glacial accumulation. In the south-east of Australia, north of Sydney, there is also evidence of the occurrence of icebergs in the sea, but that requires no revolutionary change in climate, as in 1892 icebergs off New Zealand drifted only

a little south of Sydney, and in 1894 an iceberg in the South Atlantic reached a latitude ($26\frac{1}{2}°$ S.) considerably north of Sydney.

In Gondwanaland there was a series of scattered glacial accumulations and they were not exactly simultaneous; as one of them waxed, its neighbour waned. They were all fairly near the sea, in positions where there was cold water coming from the south, and the mountains had a high winter snowfall, and a cold, cloudy summer. All those centres of glaciation may be explained by a different distribution of land and water and different elevation of the land.

There are other points to which I would have liked to refer. The three points which are clearest in the geological history of climate are: that the climate of the earth, as a whole, has remained fairly uniform throughout geological time; that there have been zones of climate, which have been, approximately, parallel to the Equator; and that the thermal isanomalies at various times have been greater than at present. Lord Kelvin, a great authority on thermo-dynamics, insisted that if Northern Asia, Northern America, and Northern Europe were submerged 1000 feet, the present ice-bound Arctic Ocean would be replaced by an iceless sea, and an island at the North Pole would have had a temperate climate, and would have been subject to frost only in the hollows. Supported by the authority of Lord Kelvin,* I would repeat, as I said in a contribution to a discussion in 1906,† that "all the variations in climate which have been established on adequate evidence can be accounted for by differences in atmospheric and oceanic circulation, due to different distribution of land and water," and different elevation of the land; and that the Gondwanaland glaciation was developed "in a number of scattered localities, where mountains occurred beside the sea, and where the meteorological conditions produced a high snowfall and a low summer temperature." All the changes which have been established can be explained without a shifting of the Poles, or a drift of the continents, or material variation in the heat supplied from the sun.

Dr. C. E. P. Brooks: I have welcomed Dr. Simpson's addition of solar radiation to the discussion of climatic variations, because I feel that it explains the facts of the Pleistocene glaciation as no other theory has done. But I think he has under-estimated the possible effect of variations of land and sea,

* "Geological Climate," 'Trans. Geol. Soc., Glasgow,' vol. v, p. 245 (1877); 'Pop. Lect. and Addr.,' vol. 11, pp. 273–98 (1894).

† "Climatic Variations, their Extent and Causes," 'Internat. Geol. Congress,' Mexico, p. 426 (1906); Ann. Rep. Smiths. Inst., p. 354 (1908).

especially the effect of ocean currents. Take, for example, the Atlantic; the average temperature of the North Atlantic in 50° to 60° N. is nearly 7° C. higher than that of the South Atlantic in 50° to 60° S., and the whole North Atlantic between 30° N. and the Arctic Circle is on the average about 5° warmer than the South Atlantic between 30° S. and the Antarctic Circle. That steady difference can only be due to the fact that there is a source of heat for the North Atlantic which is not open to the South Atlantic. That source might be winds or ocean currents, and calculations from the latest available data show that nearly the whole of that heat is due to the fact that two-thirds of the Atlantic Equatorial Current is deflected into the Northern Hemisphere, and only one-third into the Southern Hemisphere.

During the greater part of geological time old-fashioned palæogeographical reconstructions show that instead of only two-thirds, practically the whole of the Equatorial Currents which existed then were deflected into the Northern Hemisphere. That means that during those times the temperature of the oceanic parts of the Northern Hemisphere must have been decidedly higher than the temperature of the oceanic parts of the Southern Hemisphere. Even at present the thermal equator does not coincide with the geographical equator; on the average it lies in 6° N. latitude, and in the Indian Ocean it is in 15° N. Moreover, the thermal equator is not concentric with the geographical equator, and if one calculates the position of the North Pole from the thermal equator, one finds that it lies in latitude 81° N. The alteration of the position of the thermal equator disturbs the symmetry of the two hemispheres, and although the difference between the mean temperatures of corresponding latitudes in the Northern and Southern Hemispheres never exceeds 5° C., yet the Northern Hemisphere is everywhere warmer than the Southern, and one has to shift the temperature curves by 4° or 5° of latitude to get the best fit.

In the late Carboniferous the conditions were such that the diversion of heat from the Southern to the Northern Hemisphere was greater than at any subsequent time in the geological history of the earth. Palæogeographical reconstructions show a wide funnel opening to the Pacific, continuing in subtropical latitudes along the Tethys Sea into the Mediterranean and through that into the Atlantic. The Tethys Sea must have received all the warm equatorial water of the Pacific. A current in the Northern Hemisphere is deflected to the right, so that the warmest parts of that sea would be along the northern shore. Whether it was the geographical equator or not, it must have been the thermal equator. Whatever value plants may have as climatic indices, I think that in general terms they present us with the facts that in the

Upper Carboniferous the Northern Hemisphere was warmer than the Southern, and that what appears to have been the botanical equator was just where we should expect to find the thermal equator.

Finally, with regard to the ice-sheets. Dr. Simpson has pointed out that cloud is the greatest hindrance to solar radiation. I think that with a warm ocean on one side and a cold ocean on the other side, there existed the best possible conditions for low cloud and fog, and the reflecting power of cloud is so great that it is almost capable of imposing upon any place, not the climate of its latitude, but the climate of its geographical surroundings, and in particular of the oceanic currents which wash its shores.

The other aspect of the problem is that of warm polar climates, but they have been disposed of so thoroughly by previous speakers that there is very little to add. I did not know that Lord Kelvin had attacked that problem of polar ice, but Prof. Kerner, in Austria, and myself have also investigated it and arrived at the same conclusion: that a small increase of temperature, which would be given by another current flowing into the Arctic Ocean, would wipe out the ice and give a far more favourable climate, one which would be capable of maintaining a moderate vegetation everywhere.

Dr. C. Tate Regan, F.R.S.: A study of the present and past distribution of certain groups of animals throws light on the history of the earth. Fresh-water fishes are very important in this connection. Originally they may have come from the sea; but most of them belong to families, or even orders, that are restricted to fresh water and have evolved in it. Such true fresh-water fishes cannot cross the sea, and their distribution affords evidence of former land connections, or of ancient lines of severance. It is generally accepted that during the Eocene the continents were isolated and developed their own peculiar faunas, and that after the Eocene nearly the present connections were established, permitting interchange. It is interesting that the distribution of fresh-water fishes was, in the main, much the same in Eocene times as it is to-day. In North American deposits of lower and middle Eocene age occur representatives of the families characteristic of North America now; in Eocene beds of Africa are found the bones of catfishes, of a genus that is still living in Africa, but is unknown elsewhere.

It is clear, therefore, that the main distribution of fresh-water fishes was accomplished before the Tertiary; it depended on the distribution of land and water in Cretaceous times.

South America has a very large and diverse fresh-water fish fauna, which

has no relationship to that of North America; they have not a single family in common. On the other hand, the relationship between the fresh-water fishes of South America and Africa suggests that these two continents were connected in Cretaceous times. The Lepidosirenidæ—*Lepidosiren* in South America, *Protopterus* in Africa—are fresh-water fishes specialised for life in tropical swamps. The Characins are a large group of fresh-water fishes restricted to Africa and to South and Central America; they are tropical, and have not colonised Patagonia or the Cape region of Africa. These are examples of the evidence that leads to the conclusion that in Cretaceous times South America and Africa formed one continent, which had a tropical climate. Did they become two by sinking of the connecting land, or by floating away from each other? The distribution of marine fishes has some bearing on this.

The distribution of marine fishes is dependent on temperature. I define the tropical zone as limited by the mean annual surface isotherms of 20° C. There are many families of marine fishes that are restricted to the tropical zone. Most marine fishes live near the coasts, and do not cross oceans; for such coastal fishes of the tropical zone there are two main regions, the Indo-Pacific and the American; the fish-fauna of West Africa is mixed, but is mainly of Indo-Pacific affinities.

The American region of the tropical zone extends on the Pacific coast from Southern California to northern Peru, and on the Atlantic coast from South Carolina to southern Brazil. Many of the genera are peculiar to this region, and the species tend to fall into pairs, one on the Atlantic side, and its nearest relative on the Pacific side.

In Eocene times the Atlantic and Pacific Oceans were in communication, North and South America being separated by sea. After the Eocene, by the establishment of a connection between North and South America, a formerly continuous fauna was divided into two parts, and the development of two species, Atlantic and Pacific, out of one, is the amount of evolution since the Eocene. It is clear that throughout the greater part of the Eocene the peculiar American genera must have been differentiating into the species that were afterwards to divide into two. But America could not have developed a distinct tropical marine fauna unless it were well separated from other lands. It could not have been, as Wegener's map shows it, just beginning to float away from Africa in the Eocene.

I am therefore opposed to Wegener's hypothesis. From the distribution of fresh-water fishes I conclude that Africa had in Cretaceous times much the same climate as it has to-day, and from the distribution of marine fishes I

conclude that in Eocene times the Atlantic Ocean was a wide one. Africa was not much nearer to South America, nor to the South Pole, than it is now.

Dr. HAMSHAW THOMAS: In spite of all that has been said to-night, there still remain many striking anomalies in the past distribution of plants, which require explanation by some theory such as that outlined by Dr. Simpson. In my opinion Dr. Simpson's hypothesis explains some of the difficulties much more satisfactorily than any other view which has been put forward. One important omission in this discussion has been the absence of any reference to changes in precipitation. In regard to the distribution of plants, rainfall may be of far more importance than temperature. I should like to point out in this connection two things. First, in order to get the vast amount of ice covering South Africa in former periods there must have been an enormously increased amount of precipitation, which must be accounted for. I do not think that changes in the distribution of land and water alone will account for it. Secondly, when the temperate or perhaps cold-temperate plants were living in the Cretaceous or Eocene period in Greenland, we had in England a flora which is most nearly related to a modern rain forest flora. That indicates not merely a higher temperature but also a heavier precipitation.

With reference to the suggestion that some warm temperate genera may have been able to endure low temperatures in Cretaceous times, one may say that there seems to be no indication in the geological record of any gradual acclimatisation of the plants which existed in Eocene times in Europe as the Ice Age approached and the climate became colder, and, presumably, also drier.

I ask, in conclusion, whether Dr. Simpson can tell us if it is not possible that a great increase in the cloud conditions over the world and a great increase in the rainfall under conditions of an increased solar radiation would involve a large area in the Tropics having an approximately uniform temperature, or at least a temperature which varied very little between winter and summer and between day and night. I believe that if one can postulate a dense cloud belt over a large portion of the earth, say, in the Eocene period, that might account for the apparent fact that plants growing then had a much wider distribution than they show to-day. In the Jurassic period undoubtedly some of the known plants had a very wide distribution. Could there have been a heavy cloud belt over the earth and a heavy rainfall at that period also?

Dr. Simpson (in reply): As I have not time to reply to all the speakers, I propose to limit my reply to Prof. Seward's remarks. It would appear from what Prof. Seward and other speakers said that most of the evidence put forward by geologists as evidence of climate is no evidence at all. Prof. Seward tried to demonstrate that ice on the Equator was not so very surprising, and, as his method of demonstration was typical, I must refer to it. We were shown a photograph of a glacier descending into what looked like a tropical forest. From this the conclusion was drawn that we can have ice sheets surrounded by tropical vegetation, hence there is no difficulty in having an ice sheet in the Tropics. Prof. Seward, however, had to go to New Zealand for his photograph, and New Zealand is almost as near the Pole as the Equator. Then a glacier is not an ice sheet, but a highly localised phenomenon, and I said that large departures from zonal climates may be met with in small regions. The presence of a glacier near luxuriant vegetation does not prove the possibility of an ice sheet in present conditions, even in New Zealand, much less in the Tropics. When, therefore, Prof. Seward follows his New Zealand photograph with a drawing of a glaciated Gondwanaland with luxuriant vegetation, it just is not evidence. In conclusion, I would like to support Prof. Seward in his plea that geologists and meteorologists should get together to study this problem. Personally, I have received great help in what study I have given to the problem of past climates from Prof. Seward and I shall always be glad to discuss the matter with geologists.

591.392.1 : 597.8 *Rana*

Experimental Distortion of Development in Amphibian Tadpoles.

By DOROTHY E. SLADDEN, Zoology Research Department, Imperial College of Science.

(Communicated by E. W. MacBride, F.R.S.—Received March 18, 1930.)

[PLATES 25–27.]

Introduction.

In 1908, Tornier published a paper (1) on the probable causes of the formation of the abnormal "fancy" races of goldfish. All these races originated in China where the wild ancestor (*Carausius auratus*) still abounds in the streams, It had been supposed that these races were produced by a secret process known only to the Chinese breeders. The fish during winter were kept crowded in earthenware pots, on shelves in dark and insanitary huts; in summer they were transferred to small and filthy tanks overgrown with weeds. In these tanks they spawned and much of the spawn perished; amongst the fraction which survived, however, all sorts of abnormalities were found. By selecting the most striking of these, the breeders secured the parents of their "fancy" breeds, which showed in every succeeding generation a strong tendency to revert to the normal; only by the most rigid selection was anything like a "pure" race obtained.

Tornier drew the conclusion that the abnormalities were due to the effects of lack of oxygen in very early stages of development. This lack induced what he called "plasma-weakness" in different parts of the formative area of the very young embryo. In consequence of these localised areas of weakness the protoplasmic part of the egg was liable to mechanical distortion, induced by abnormal pressure from the yolk; the latter absorbed water and swelled, thus crushing and destroying the protoplasmic structures from which the future organs of the adult are formed.

These abnormalities once produced were, according to Tornier, hereditary, but were not transmitted to the next generation as a "factor" or "gene" for a specific abnormality (*e.g.*, a double tail or a bulldog face) but as a degree of plasma-weakness, which in every generation produced mechanically the same morphological results. If, however, the race was replaced into a normal environment the weakness tended to disappear after a certain number of generations.

Tornier attempted to test these conclusions by performing experiments with the eggs of a large newt, the Axolotl (*Siredon pisciformis*). He subjected the eggs to darkness, which prevented the plants in the water from producing oxygen, and then placed them for 48 hours in a 5 to 10 per cent. solution of sugar, which he assumed would act as an absorbent of any oxygen present in solution. In this way he obtained embryos which exhibited all the phenomena of "plasma-weakness." The axis of the embryos in question had been shortened and in some cases bent inwards in a concave curve, the head in consequence bent upwards and the mouth cavity and gill-slits enlarged. He succeeded in rearing a few of the embryos to an advanced larval stage. The larvæ had swollen abdomens, like those of the "Ranchu," a race of fancy goldfish. When these abnormal specimens were dissected it was seen that the abdomen was swollen with water, and that the liver had consequently been impeded in its growth and reduced to less than a quarter of its normal size.

By applying his methods to frogs and toads Tornier was able to get further results. In these later cases he used 25 per cent. solution of sugar for a shorter time. The yolk plug which normally fills the blastopore became enormously enlarged and prevented or greatly impeded the growth of the tail.

Berndt (3), who was attracted by Tornier's views, experimented with the "fancy" breeds of goldfish and endeavoured to show that the marks of their races were not due to general weakness, as Tornier supposed, but to a specific weakness separately inheritable. Berndt confirms Tornier's idea that life in aquaria leads to rapid degeneration of stock, but he does not agree with the latter's theory of "yolk pressure," as he found the swelling of the body-cavity might only appear in late stages, from which yolk was absent, the young forms showing no signs of it, and the goldfish with swollen abdomens (characteristic of certain breeds) appear to owe the swelling to enlarged ovaries and fat masses, and not to fluid.

At Prof. MacBride's suggestion I repeated Tornier's experiments in 1928, using the eggs of the common frog (*Rana temporaria*) and subjecting them, at the end of segmentation, to a 10 per cent. solution of sugar for 4 hours. At the time of hatching only a few of the larvæ were abnormal, the principal type of abnormality being the swollen body-cavity. Without exception these larvæ died shortly after hatching, leaving only those which appeared perfectly normal. These proceeded to develop until just before the hind limbs were due to appear (about 8 weeks after hatching), when in a few cases a bend appeared at the base of the tail. These abnormalities were followed by others appearing at intervals during the next four months, showing the same type of deformity.

During the following year, 1929, experiments were again carried out with the eggs of *Rana temporaria*, this time subjecting them to reduced air pressure. This experiment gave the same results as that of the previous year, the majority of abnormals appearing as before at a fairly advanced stage of development. In this case, however, a larger percentage of tadpoles were affected in their advanced stages. Another batch of eggs was treated with 25 per cent. sugar solution this year, for the same length of time—namely 4 hours. Also the experiment of the previous year, using 10 per cent. sugar solution, was repeated.

The table given below shows the percentage of abnormalities obtained in 1929, from the three experiments described above, at time of hatching and 5 months later. In the latter case, the percentage is of the total number of living larvæ, and not an additional percentage to that taken at time of hatching. As already stated, the early abnormals died during the first month of larval life.

Table.—Percentage of Abnormalities, 1929.

	Reduced air pressure.	Control.	10 per cent. sugar solution.	Control.	25 per cent. sugar solution.	Control.
	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.	Per cent.
At time of hatching	0·8	3·5	3·2	2	10	0·7
5 months later	9	0·0	6	0·0	2	0·0

Technique.

In 1928 several artificial fertilisations were carried out with the eggs of *Rana temporaria* previous to treatment with sugar, but the best results were obtained from a pair of frogs in which the eggs were directly fertilised. In this case the larval death-rate was considerably reduced, and therefore these eggs were used for the following experiment.

The spawn, 24 hours after fertilisation, was divided into two, one half being subjected to a 10 per cent. solution of cane sugar in tap-water for 4 hours, and the other half kept under normal conditions to act as control. The sugar solution was stirred at frequent intervals during the time it contained the spawn, to maintain an equal concentration. After 4 hours the spawn was rinsed in clean water and transferred to running water until the larvæ hatched. The newly hatched larvæ were transferred to aquaria containing "still" tap-water and the necessary conditions for their development during larval life. After metamorphoses the young frogs were kept in vivaria.

The following year (1929) directly fertilised spawn was again used. Three sets of experiments were carried out. In the case of the first set, the spawn, 24 hours after fertilisation, at the same stage of development as in the previous year, namely, at the end of segmentation, was kept in distilled water, in an atmosphere which was maintained at 300 mm. (of mercury) below the atmospheric pressure at the time, for 4 hours by means of a water-vacuum pump. The eggs were then transferred to "still" tap-water and allowed to develop under the same normal conditions as the control.

The experiment with 10 per cent. sugar solution of the previous year was repeated, and in addition a 25 per cent. sugar solution was used on another batch of eggs. The technique of the second and third sets of experiments was the same as that of the previous year. In all three cases some of the eggs were used as controls.

How far treatment with sugar solutions, or exposure to an atmosphere of reduced pressure, actually influences the oxygen consumption of the eggs—as claimed by Tornier—remains to be determined. For immediate purposes, however, it is more important to know precisely the results of such treatment than the precise nature of their underlying causes.

Results.

When the eggs treated with 10 per cent. sugar solution hatched, the greater percentage of larvæ were normal. Some died immediately on hatching, but of the survivors a few showed marked abnormalities of a definite type, namely, the swollen body-cavity described by Tornier (1 and 2) and Berndt (3) (Plate 25, fig. 2). In a few cases this was accompanied by a bend at the base of the tail (Plate 25, fig. 1). When these larvæ with the swollen abdomens were sectioned, it was found that the yolk in the gut had expanded—possibly due to the absorption of water as Tornier affirmed—rupturing the walls of the gut and extruding into the body-cavity (Plate 27, fig. 9). In some cases the body-cavity was also swollen, the liver and pronephros being considerably reduced. With few exceptions these larvæ died shortly after hatching, and those which survived did so only until their supplies of reserve nutriment were exhausted, when, being unable to feed (due possibly to a defect of the alimentary canal, or, owing to the distention of the body-cavity in cases in which it occurs, it was not possible to bring the mouth in contact with the food), they also died.

Without exception the newly-hatched abnormal larvæ were seen to lie on the floor of their tank instead of attaching themselves to submerged objects in the usual way by means of the adhesive organs or suckers. This is due to

the fact that these organs are considerably reduced in size in an abnormal larva (Plate 27, fig. 11) as compared with those of a normal one of the same age (Plate 27, fig. 12). In addition to this fact, the adhesive cells are either entirely absent or, if present, not sufficiently well developed to penetrate the epidermis and so reach the exterior. The adhesive organs are therefore rendered functionless.

After the above-mentioned abnormal larvæ died, there remained only those which to all external appearances were perfectly normal at the time of hatching. These continued to develop in the usual way, over a period of 8 weeks, until a fairly advanced stage of development was reached, namely, that at which the hind limbs appear. At this time one or two of the larvæ developed a slight tendency to swim in a circle, and on being examined more closely the cause was found to be a bend at the base of the tail, similar to that observed in the younger stages, but to a lesser degree. Such abnormalities as these continued to make their appearance during the next 4 months. The bend in some cases flexed the tail to the right and in others to the left.

As time went on and more individuals appeared showing the same defect, it became clear that the longer the period between the time of hatching and the first external evidence of the bend, the greater was the defect (Plate 25, figs. 3 and 4). Thus the frogs shown in figs. 6 and 8 (Plate 26) with extreme abnormalities were 5 months old before any trace of the defects became apparent in the tadpole.

When one of these later larvæ was sectioned (Plate 27, fig. 13) it was found that on the inner or "concave" surface of the bend, the muscle bundles forming the myotomes were considerably shortened and irregularly arranged, while on the other surface the myotomic formation was perfectly regular and the muscle bundles of normal length. The younger stage in which the bend was evident at the time of hatching showed a similar internal defect in the muscle bundles in the region of the bend (Plate 27, fig. 10).

Towards the end of larval life, in the case of the later stages described in a previous paragraph, the sacral region of the tadpole commenced to show signs of distortion in relation to the bend in the tail. This gave rise in a newly metamorphosed frog to a dorsolateral projection or "hump" on the opposite side of the body to the bend. In cases in which the tadpole's tail was flexed to the right, the distortion was on the left side of the body of the metamorphosed frog.

The extent to which this distortion was carried varied, as already stated, in accordance with the age of the larva. When a period of 5 months or over

elapsed between the time of hatching and the first appearance of the bend, the distortion was accompanied by a twisting of the hind limb or pelvic girdle away from the projection (Plate 26, fig. 6). This had the effect of displacing the hind limbs, the left one becoming almost central and the other in contact with the right fore-limb, and *vice-versa*. This defect prevented the young frog from feeding.

In one case (Plate 26, fig. 8) the twisting of the pelvic girdle was sufficient to displace the left limb, preceded by the anus, to the larva's right side; the right limb was suppressed altogether, an effect which may be due to compression in growth at an early and critical period of development. The projection, which in this case was towards the larva's left side, was flattened at its apex and in the young frog was often brought into contact with the ground, when in a resting position, to act as a support. This frog was unable to feed itself owing to the same reason given above. Its movements were even more affected, as the only means of progression was by a series of shortened leaps (always in an oblique direction) which greatly exhausted it, and often caused loss of balance. As in the previous case, death followed closely on metamorphosis.

A section (Plate 27, fig. 14) transverse to the body and through the sacral region of the frog (see Plate 26, fig. 7) appears to have three distinct notochords intercepted by cartilage, but in a reconstruction of the same larva this was clearly due to the manner in which the notochord had become distorted. It was seen to be prolonged up into the projection in the sacral region and then passed back again to the median line of the body, thus becoming almost bent on itself.

Twenty-six weeks after fertilisation a new type of deformity made its appearance among the few apparently normal larvæ in which metamorphoses were not completed. This took the form of a defect in the articulation of the femur with the tibio-fibula, resulting in a stiffening of the affected limb (Plate 26, fig. 5). In every case the hind limbs only were affected. This larva died 4 days later. Three other abnormalities of the same type followed, with either right or left limbs affected, and one with both. These four metamorphosed successfully.

Owing to the scarcity of the material it was decided not to sacrifice any of the young frogs in order to examine the internal structure of the affected limb, but this it is hoped will be done at a later date.

The above described abnormalities are those obtained from the experiment with 10 per cent. sugar solution in 1928. Those resulting from the experiments in the following year showed exactly the same types of abnormality, the only

difference being the percentage of larvæ affected according to the nature of the experiment, as shown by the table on p. 320. The percentage of abnormalities at the time of hatching, as seen by the table, was highest in the case of the larvæ treated with 25 per cent. sugar solution and lowest in the case of the larvæ subjected to reduced air pressure. Five months later, however, this condition was reversed.

No cases of stiffened limbs have been obtained this year (1929) by any of the methods employed.

Summary.

Eggs of *Rana temporaria* were exposed, at the end of segmentation, 24 hours after fertilisation, to a 10 per cent. solution of sugar in tap-water for 4 hours; they were then transferred to normal aerated water. The resulting larvæ exhibited marked structural abnormalities, although these might not be obvious for a prolonged period, *e.g.*, 3 to 4 months after fertilisation. These abnormalities have been described as (*a*) distention of body-cavity; (*b*) rupture of gut and extrusion of yolk; (*c*) flexure of tail; (*d*) distortion of sacral region; (*e*) non-appearance of limb.

In conclusion, I would like to express my deep gratitude to Prof. E. W. MacBride not only for the great encouragement and assistance which he has given me while I have been working under his supervision, but also for his original suggestion that this line of research could be profitably followed. It is improbable that this work would have reached a successful conclusion without help which Mr. H. R. Hewer, M.Sc., has so freely given me.

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(2) Tornier, G., " Über experimentelles Hervorrufen und Naturentstehen von Mopsköpfen, Cyclopen und anderen Vorgebürtlichen Kopfverbildungen bei Wirbeltieren." 'Berlin Sitz. Ber. Ges. Natf. Freunde,' pp. 298–315 (1908).

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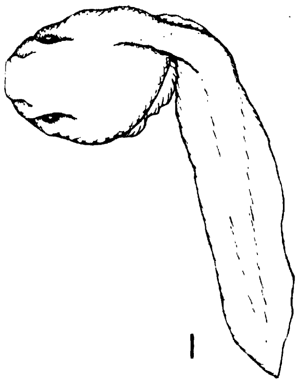
DESCRIPTION OF PLATES.

The illustrations on Plates 25 and 26 are of tadpoles and frogs from eggs treated with 10 per cent. sugar solution in 1928. The body-length is that of a straight line drawn from the tip of the nose to the base of the tail. Age taken from time of hatching until fixed.



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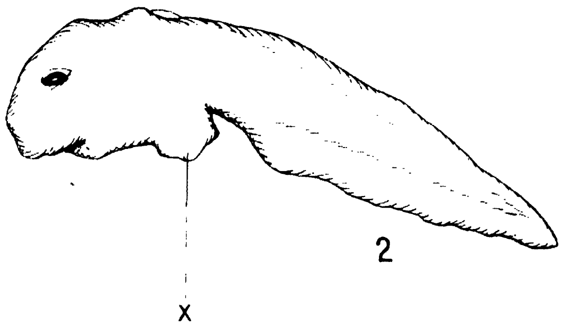

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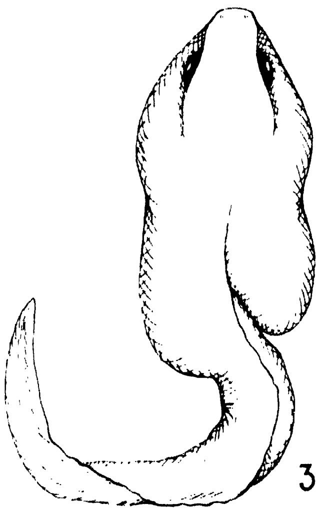

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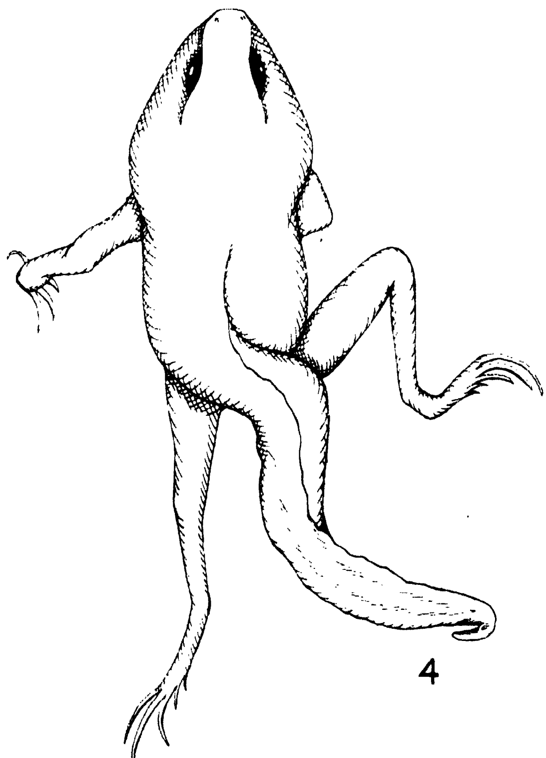

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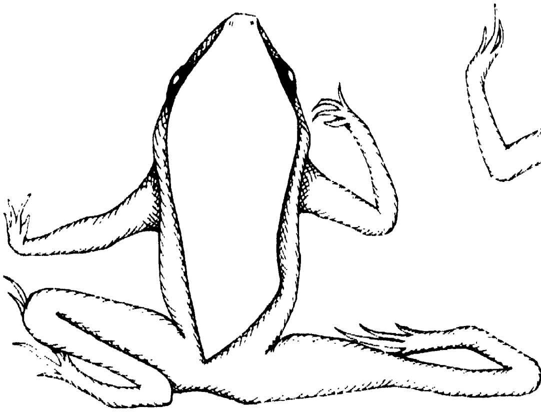

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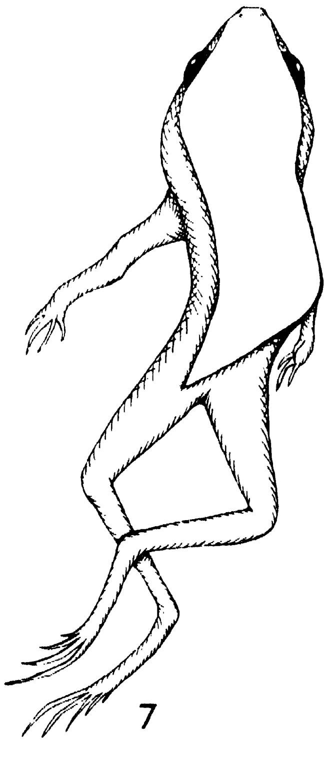

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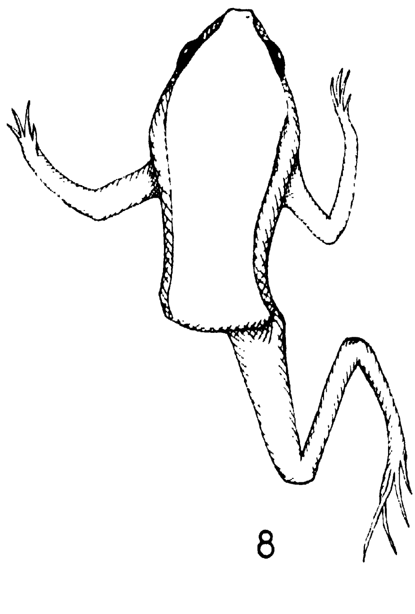

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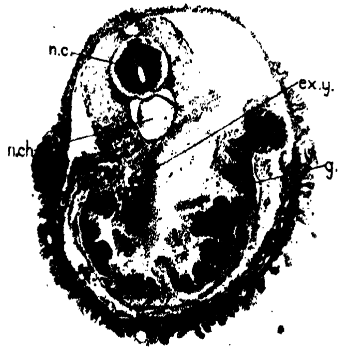

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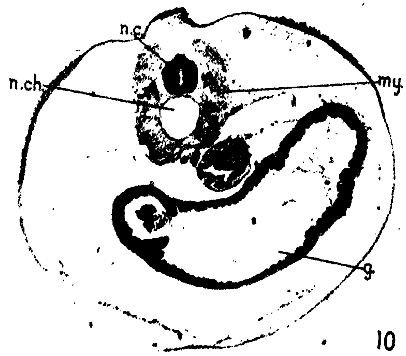

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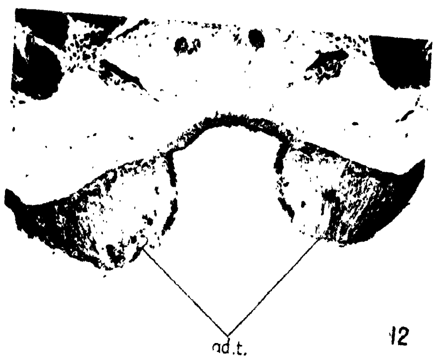

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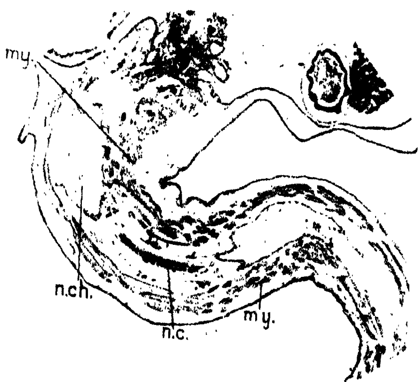

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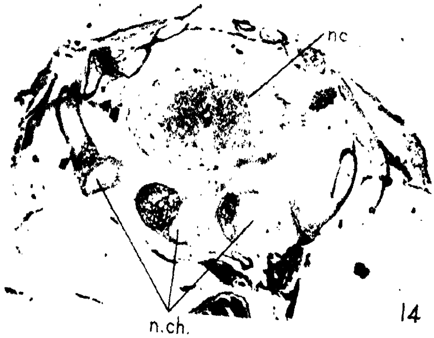

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PLATE 25.

FIG. 1.—A tadpole showing flexure in tail owing to deformation of myotomes. Age 5 weeks and 2 days. Length of body, 3 mm.

FIG. 2.—A tadpole showing distention of body-cavity, X. Length of body, 8 mm.

FIG. 3.—Limbless tadpole, age 20 weeks, showing flexure in tail. Length of body, 10 mm.

FIG. 4.—Tadpole with four limbs present. Note the stiffening in the left hind limb and flexure in tail. Body length, 11 mm. Age, 20 weeks and 2 days.

PLATE 26.

FIG. 5.—Metamorphosed frog showing stiffness in right hind limb, and slight distortion of sacral region. Age, 11 months. Length of body, 17 mm.

FIG. 6.—Metamorphosed frog. Length of body, 10 mm. Note distortion of sacral region and displacement of hind limbs. Age, 26 weeks.

FIG. 7.—Metamorphosed frog showing distortion of sacral region. Length of body, 9·5 mm. Age, 26 weeks.

FIG. 8.—Metamorphosed frog. Note twisting of sacral region and absence of right hind limb. Length of body, 10 mm. Age, 22 weeks.

PLATE 27.

FIG. 9.—Microphotograph. Trans. sect. of tadpole shown in Plate 25, fig. 2. Note the extrusion of the yolk into the body-cavity. × 43. *n.c.*, nerve cord; *n.ch.*, notochord; *w.g.*, wall of gut; *ex.y.*, extruded yolk.

FIG. 10.—Microphotograph. Trans. sect. through the sacral region of a 10-mm. tadpole showing a defect in the myotomes. × 32. *n.c.*, nerve cord; *n.ch.*, notochord; *g.*, gut; *my.*, defect in myotomes.

FIG. 11.—Microphotograph. Vert. sect. through sucker of an abnormal tadpole (*cf.* fig. 12). Note absence of adhesive tissue on surface of suckers. × 90.

FIG. 12.—Microphotograph. Vert. sect. through suckers of a normal tadpole (*cf.* fig. 11). × 90. *ad.t.*, adhesive tissue.

FIG. 13.—Microphotograph. Frontal sect. of a tadpole showing deformation of tail myotomes. × 24. *n.c.*, nerve cord; *n.ch.*, notochord; *my.*, defect in myotomes.

FIG. 14.—Microphotograph. Trans. sect. of frog shown in Plate 26, fig. 7. Note the notochord sectioned in three places owing to distortion of the sacral region. × 28. *n.c.*, nerve cord; *n.ch.*, notochord.

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Numbers and Contraction-Values of Individual Motor-Units Examined in some Muscles of the Limb.

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(Received March 27, 1930.)

[PLATES 28–30.]

CONTENTS.

" The muscle with its nerve may be thought of as an additive assemblage of motor-units, meaning by motor-unit an individual motor nerve-fibre with the bunch " [or " squad " (E. L. Porter, 1929 (1))] " of muscle-fibres it activates." (2) The components of such a unit can claim sufficiently close and sufficiently analysed interrelation to warrant acceptance for many purposes as a single functional entity. In application to reflexes, the unit thus resulting favours brevity and directness of quantitative statement. Its correspondence with a so-to-say quantum reaction, which forms the basis, by combinations temporal and numerical, of all grading of the muscle as effector-organ, fits

it for measuring that grading. It is, moreover, applicable centrally as well as peripherally, since the motor-units active number the motoneurones discharging. Such mensuration, the total of the pool of motoneurones being known, evaluates *per se* the given reaction in terms of the total potential reaction.

1. Contraction-Tension of the Individual Motor-Unit.

In the following experiments it was therefore sought to find the physiological size of the motor-unit, *i.e.*, to measure its contraction-tension. The muscles examined (cat) have been *gastrocnemius* (*median head*) *soleus, semitendinosus, extensor longus digitorum*, and, less fully, *tibialis anticus* and *crureus*.

The motor-units composing a muscle will present for determination an average value and particular individual values.

I. *Average Contraction-value.*

The contraction-tension yielded by a muscle under maximal stimulation of its nerve when divided by the number of motor-fibres in the nerve will give the average contraction-tension of the individual motor-units.

i. *Procedure.*

(1) *Removal of the Afferent Fibres from the Muscle-nerves.*—The requisite root-ganglia were excised to give degeneration of the afferent fibres in the muscle-nerves. For removal to be complete, allowance has to be made for the individual variation of segmental nerve-supply so common with the muscles of the hind-limb. For *gast. med.*, though usually only a bi-segmental muscle, the ganglia excised were 6th,* 7th, 8th and often 9th. *Soleus* was dealt with similarly. In no experiment was a supply from 9th segment in fact found either to *gast. med.* or *soleus*. For *semit.* the ganglia excised were 6th, 7th and 8th ; for *ext. l. dig.* similarly, with in some instances 5th as well. In the few observations on *tib. ant.* and *crureus* the 5th ganglion was always included along with 6th, 7th and 8th.

In operating for excision of the ganglia the spinal theca was not opened. The extra-thecal length of these roots, though less than in the dog, allows even where shortest, *i.e.*, at 5th, free exposure and cutting of the dorsal root proximal to the ganglion without endangering the ventral root. For exsection of the ganglion the plan has been to make with a fine cataract knife a shallow cut transversely across the dorsal aspect of the segmental nerve just distal to the

* These numbers used for the spinal ganglia proceed backward from the first lumbar segment, counted as 1.

distal tip of the ganglion; then with scissors to sever the dorsal root in its sheath some 3 mm. proximal to the ganglion. The junction of this root-stump with the ganglion was then seized with fine toothed forceps. Under the gently lifted stump and ganglion the fine knife next proceeded to dissect the ganglion free from the ventral root. This freeing was begun proximally; there it presents no difficulty or risk to the ventral root; but at its distal end the ganglion is attached to the ventral root more closely, especially so in the 8th and 9th ganglia. The operation requires full uninterrupted view of each ganglion to beyond its distal tip. The main impediment is venous bleeding (from the extensively opened bone) interrupting the view.

When securing complete ablation of the ganglion infliction of some damage on the ventral root is often unavoidable. This is much preferable to risk of incomplete removal of the ganglion. The former by subsequent degeneration of the injured fibres supplies its own numerical control; the latter not.

The ganglia on removal were placed in formol 10 per cent. for microscopic examination by serial sections. All operations were under full anæsthesia, with strict precautions for asepsis. The wound healed usually by end of the first week.

Meanwhile microscopic examination went forward with the excised ganglia. Experience showed that contrary to expectation the microscopic examination was competent to decide whether or no the ganglion had been excised completely. The proximal two-thirds were easily controlled; it was the distal tip which was likely partially to escape removal. Control specimens showed that, followed distally in serial sections 10 μ thick, the ganglion-cells at the distal end of the ganglion dwindle rapidly in number and then, somewhat abruptly, fail altogether. Absence of cells for eight successive sections guarantees the end of the gangion. The final cells are both large and small as in the ganglion elsewhere, and may lie together or scattered, and deep or superficial or both. With the distal ends of the operatively excised ganglia when the last 12 or more of the 10 sections were free from ganglion-cells the excision was accepted as complete. The ganglia as operatively excised have often shown at their distal ends entire absence of ganglion-cells for more than 100—sometimes more than 150—successive sections.

After microscopic assurance had been obtained that the operative ablation of the ganglia had been complete there followed a further necessary waiting period to ensure degenerative disappearance of the afferent fibres from the muscle-nerves.

(2) *Myographic Examination.*—Completion of the degenerative period was

succeeded by the myographic test. The fully anæsthetised animal was decerebrated back to the posterior *colliculi*, through a trephine hole with negligible hæmorrhage or disturbance of respiration. The anæsthetic was then remitted, and the limb with its required muscles was prepared by nerve-section, tendon-isolation, etc., for fixation and attachment to the myograph. *Gast. med.* is a powerful muscle, and the rigidity of the solid myograph table was for the later experiments improved by an iron plate screwed over part of its top. The torsion-wire myograph was of the mirror pattern giving high magnification ($\times$ 600) and good isometry. The torsion-wire in the great majority of the experiments had the usual friction-bearing at one end. Later, to escape all frictional damping, instead of the torsion-wire a steel torsion strip as used by Hartree and Hill (3) was employed, with mirror recording as before. In the most recent observations the torsion-wire was reverted to, the friction bearing being replaced by a knife-edge bearing on polished steel, (4) as suggested to us by the National Physical Laboratory. We tender our grateful thanks to the Director, Sir Joseph Petavel, and to Mr. Hyde for kind and valued help in this matter. The mirror-recording and magnification of tendon movement remained as before. The myograph, entirely free from the table except for the muscle whose bony attachments were clamped to the table, was carried by an iron stanchion fixed in the concrete floor and to an iron transom above, as described elsewhere. (5) Each muscle for its examination was freed from all others in respect of its mechanical effort. The details of fixation were, of course, different for the different muscles. *Soleus* was carefully dissected free from *gast. lat.* Local impairment of blood circulation was avoided. Care was taken that the stimulation of the muscle's nerve should be maximal, with sufficient frequency of stimuli to ensure good tetanic fusion, *e.g.*, somewhat above 50 per second. The muscle-nerves, deafferented, were stimulated in continuity, *i.e.*, uncut. In the earlier experiments the fellow muscles of the unoperated side were myographed for comparison. During the experiment, tests of the specified muscle-nerves for reflexes with negative result afforded useful corroboration as to whether the right roots had been destroyed, but verification by actual *post-mortem* identification was an indispensable guarantee.

(3) *Examination of the Nerves.*—On completion of the myographic tests the muscle-nerves with controls were removed at once from the bled animal, "splinted" on filter paper, and placed in osmic acid 0·5 per cent. for 12 hours. The motor nerve-fibre of the motor-unit being myelinate our enumerations have not comprised other than myelinate fibres. After thorough washing

and transference to 33 per cent. alcohol they were in part run rapidly through the alcohols and xylol and imbedded in wax for cross-sectioning, and in part mildly dissociated in weak alcohol and glycerine for three weeks for teazing.

For enumeration of the motor-fibres in cross-sections of osmicated nerve it was important that the débris of the degenerated afferent fibres should have passed beyond the stage of containing "ellipsoids," so easily confused in cross-section with sound fibres. Ellipsoids become shorter as well as less numerous as post-degenerative absorption proceeds. A short ellipsoid in cross-section can usually be distinguished from a normal fibre by following it in serially successive sections. In the muscle-nerves of the cat we found ellipsoids no longer a difficulty after the sixth week of degeneration. The shortest degeneration interval allowed was 45 days, the longest 82. Well fixed and stained with osmic acid the material was not counterstained in any way; counterstaining the sections yielded less clear photographs.

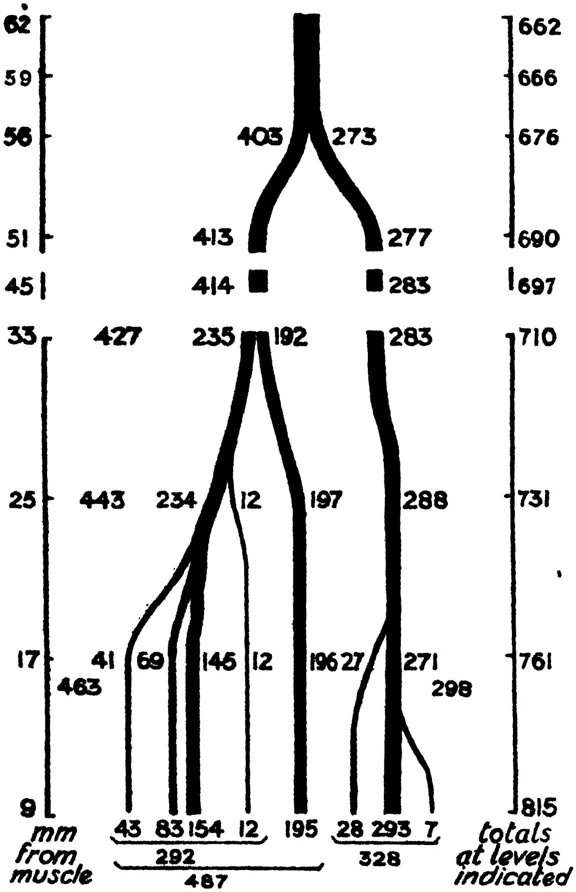
The thickness of the cross-sections used for counts was 4–5 μ. The plan finally pursued for the actual enumeration of the sound fibres was adopted only after a number of preliminary trials of several methods. One was the photographic plan successfully followed by Dale (6) (1900) in his carefully checked counts of fibre-numbers in spinal roots (cat, rat); the enumeration is made by pricking off fibre by fibre in the photograph assisted by the microscopic specimen at hand. This method we found excellent for normal nerves and owe to it some enumerations that follow. For nerves undergoing degeneration it was less suitable. In them the particles of osmic-stained débris still present in the young sclerotic tissue between and around the normal nerve-fibres forms a difficulty. Our photographs, although ranging up to $\times$ 1200 magnification, did not resolve this punctately blackened material sufficiently. Resort with the 2 mm. objective to the actual specimen had to be so frequent that danger of confusion arose for the enumeration.

The plan ultimately adopted was to use a binocular microscope, one ocular of which, furnished with an engraved diaphragm, divided the central part of the microscopic field into 25 squares each of 40 μ side, under the magnification employed. This squared area was brought successively over the whole cross-section of the nerve by a mechanical stage, the rulings of the ocular being set to correspond accurately with the axes of movement of the stage. On paper ruled with squares the cross-section of each nerve-fibre was entered correspondingly with its place in the squared field of the microscope. Fibres of large, medium, and small diameter were entered as large, medium, and small circles, respectively, making any particular fibre easy to refind. A map thus

obtained was checked by repetition from a different starting point; also, in a number of instances, by reworking over it with a micrometer in the ocular of the other tube of the binocular, and entering on the map against each fibre its diameter in *micra.* Every fibre was thus re-identified and checked. The fibres were finally counted by pricking them off in the maps, one map controlling another, occasionally supplemented by photographs as well.

ii. *Results.*

(1) *Efferent Fibres, number of; splitting of.*—Early in the work counts from different sections of one and the same nerve were frequently so discrepant that the counting was thought unreliable. This led to renewed checking, control by photography, by micrometer map-making, and in other ways.



TEXT-FIG. 1.—Normal *gastrocnemius medialis* nerve: increase in number of its nerve-fibres during 53 mm. of its course towards its muscle.

It became clear that the discrepancy was not for one and the same section, but for different sections of one and the same nerve, and that counts taken nearer the muscle gave larger numbers than those taken further away. This is illustrated by text-fig. 1 and Table I, from normal *gast. med.* nerves containing both afferent and efferent fibres.

The fact has been already noted for the nerves of the hind-limb of the frog by Dunn (7) (1900) working in Donaldson's laboratory; and by Willems (8) (1911) in the muscular branches of the trigeminus (rabbit). Recently (1927) it has been observed by McLean (9) from counts of the fibres in the nerves of the ocular muscles (dog).

Table I.—Normal (afferent-efferent) *gast. med.* nerves (cat), counted by photographic method (Dale, 1900 (6)).

——	About 40 mm. from muscle.	About 10 mm. from muscle.
Right leg	Bundle 1 572 ,, 2 311 (total 883)	Bundle *a* 265 ,, *b* 89 ,, *c* 67 ,, *d* 19 ,, *e* 17 ,, *f* 170 ,, *g* 315 (total 942)
Left leg	One bundle 795	Bundle *a* 9 ,, *b* 56 ,, *c* 144 ,, *d* 269 ,, *e* 163 ,, *f* 212 (total 853)

Important for us was whether this distal increase in the nerves involves their motor-fibres. The muscle-nerves were therefore examined 6–10 weeks after deafferentation, so that motor-fibres only were left. The countings clearly showed that the number of the motor-fibres increases as traced distally. Thus:—

Table II.

—	Millimetres from muscle.	Number of motor-fibres.	Millimetres from muscle.	Number of motor-fibres.
Gast. med.—				
Experiment 1	about 45	431	about 8	484
,, 3	,, 50	388	,, 6	459
,, 5	,, 45	448	,, 10	481
,, 26	,, 40	177	,, 15	188
Semit.—				
Larger nerve of experiment 13	,, 40	473	,, 10	512
Soleus—				
Experiment 27	,, 25	141	,, 10	152

Dunn (7) (1909) in observations on a frog observed similarly that the increase in number of nerve-fibres involves the motor-fibres. The ventral spinal roots of one sciatic of the frog were cut; eight months later the fibres of the cut side, counted, showed distally a percentage excess over those of the parent stem not widely different from that observed on the normal side, where efferent as well as afferent were present. The inference was that the peripheral increase in the frog concerns both afferent and efferent fibres in about equal proportions.

Our next step was to search the deafferented muscle-nerves for the implied splitting fibres. Exhaustive teazing of mildly dissociated material was eventually rewarded by discovery of some fibres actually in dichotomy (figs. 3, 5, 6). That *after* entering a muscle the myelinated nerve-fibres branch is well known. That in some cases they do so shortly before entrance has been shown for *tenuissimus* (Adrian (10)) and *sartorius* (Cooper (11)) of the cat. Our own findings extend the incidence of their splitting to nerve-regions further from muscle, and to all the muscle-nerves we have examined. We have found the splitting fibres not only as sound in the deganglionated nerves but as degenerated (fig. 7) after exclusive severance of the ventral roots. For success in the latter case the degenerative period has to be curtailed to its quite early stage, *e.g.*, 5–7 days. The degenerative process, attacking the Ranvier nodes, early breaks the junction between (fig. 7) daughter-fibres and parent-fibre. Nothing is then left to show that the fibres arose by twinning.

With approach to the muscle the rate of multiplication of the fibres within the nerve increasingly progresses. This is so in the normal afferent-efferent nerve, *e.g.*, normal *gast. med. n.*, text-fig. 1, *v.s.*; and also when the

nerve has been reduced to its purely motor-fibres by removal of all afferents. Thus :—

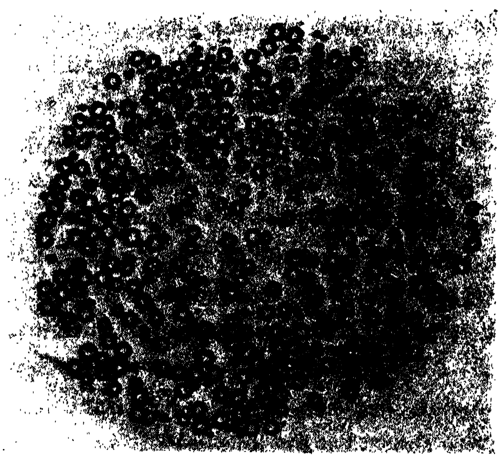
Gast. med. n. (deafferented 48 days).

At 60 mm. from muscle	....	393 fibres
,, 30 ,, ,,	....	409 ,,
,, 10 ,, ,,	....	445 ,,
,, 6 ,, ,,	....	482 ,,

For correct estimation of the motor-unit value it is clearly essential that the fibre-counts be made as far away from the muscle as practicable.

In the earlier, *i.e.*, more proximal, region of their course the fibres divide infrequently, perhaps not at all. With the relatively long nerves of *gast. med.*, *semitend.* and *soleus* the counts obtained proximally should but slightly (if at all) overestimate the original number of motor-fibres issuing to each muscle from the spinal cord. With nerves allowing examination only at shorter distance from the muscle, *i.e.*, of *ext. l. dig.* and *tib. ant.*, liability of complication by fibre-multiplication in the nerve is greater. The influence of such complication will be, of course, toward making too small the size of the motor-unit.

(2) *Efferent Fibres, Sizes of.*—Before passing to the actual "average" values found, a question rises concerning the significance of such "averages" in the present case. When freed from commingled afferents the motor-fibres of a muscle in their collective purity show somewhat surprising diversity of



TEXT-FIG. 2.—Nerve-trunk of *gast. med.* × 160; afferent fibres removed by 45 days degeneration; efferent fibres, 448, gave on stimulation 15,800 gm. tetanic tension in the muscle. Experiment 5 of Table VI., p. 348.

size. In one and the same nerve they can range from 20 μ down to 2 μ. Such diversity suggests diversity in the corresponding motor-units. That suggestion demands as a preliminary question whether or how far the smaller fibres of the revealed admixture are in fact of direct provenance from the motor spinal roots. For this further experiments were made.

(3) *Possible Sources of the Small Myelinate Fibres.* (*a*) *Ventral Spinal Roots.*—If these small fibres are of direct provenance from the motor spinal roots, not only should they remain sound after excision of the dorsal root ganglia, but they must degenerate after section of the ventral roots. In order to sever the ventral roots without risk of damage to the dorsal or their ganglia the ventral roots were cut without opening the spinal theca, and so far proximal to their junction with the dorsal root and ganglion that not only were the ganglia not injured, but they were hardly seen. Degeneration periods of 5½, 6 and 7 days were allowed, in order to catch degeneration in the smallest fibres. The muscle-nerves (osmic and teazed in 50 per cent. glycerine) showed among their degenerating fibres a number of fine ones, down to 3 μ (unshrunken by alcohol, etc.) and less. Many fine fibres, it is true, remained sound (from the dorsal root ganglia), some of them being slightly smaller than any of the degenerated (ventral root) fibres.

It was clear therefore that many fine myelinated fibres down to 2·5 (alcohol) in these muscle-nerves belong to the fibre-outflow of the ventral spinal roots. This conforms with the abundance of similar fine fibres remaining sound after excision of the dorsal root ganglia. Whether the ventral root accounts for *all* these latter remained, however, still a further question. For this additional experiments were made.

(*b*) *Sympathetic Chain.*—Many post-ganglionic sympathetic fibres of this region are, in the cat, myelinated (Langley (12)) though thinly, and are of 3 μ, some even of 6 μ diameter. The small fibres persisting in the muscle-nerves after ablation of the root ganglia might conceivably be some of these. The abdominal sympathetic chain with its ganglia, 5–8 inclusive, was therefore excised.

Trial observations on degeneration of the small myelinate fibres of the cervical and abdominal sympathetic had shown that 4½ days give clear degeneration pictures (heavy osmication followed by glycerine dissociation and examination by teazing), and that 8 days degeneration, in the case of such fine fibres, may leave, by the osmic method, little trace of their degeneration, absorption having cleared up the débris.

Three experiments were made with periods of 4½, 6 and 7 days respectively; in the sacral sympathetic stump degeneration was abundantly plain in all. In the

exhaustively teazed muscle-nerves which included *semimembranosus* and *biceps* as well, besides the larger fibres, which were all of them sound and in which no degeneration was to be expected, some three thousand myelinated fibres from 6 μ downward must have been looked at and all found perfectly sound. High up in the nerve of *gast. med.* of the sixth day experiment, however, two thin myelinated fibres of 2–3 μ were unmistakably degenerating. No single other degenerate fibre was seen.

Also two converse experiments were made. The combined ventral and dorsal spinal roots 6–9 inclusive were cut just distal to their ganglia.

Twelve and 16 days were allowed for degeneration. The heavily osmicated and gently dissociated muscle-nerves were then exhaustively teazed. All their fibres, large and small, were obviously degenerated, except for the following: two 2–3 μ fibres in *semit.* nerve, and two closely like them in *gast. med.* nerve. These fibres lay not in the sheath of the nerve; the sheath was always teazed apart and separately searched. They lay actually amid the other nerve-fibres. All four were rather questionably, *i.e.*, imperfectly, myelinate. They resembled fibres of similar size numerous in the lumbar sympathetic chain. It may be objected that spinal nerve severance close distal to the ganglia will injure the grey ramus itself and therefore few if any of its fine myelinate fibres will remain, and that the above four were such. But if so, after complete extirpation of the root ganglia, a trespass which goes at least as far distal, there should follow in the muscle-nerves—if these fine fibres do *not* come from the ventral roots—a complete degenerative disappearance of fine myelinate fibres. But the charts show that on the contrary they remain numerous, thousands as compared with the above four.

The four fibres and the pair in the converse experiments were perhaps post-ganglionic sympathetic (22), which retained their thin myelin sheath unusually far distally. Evidently the contribution of myelinated fibres from the sympathetic chain and its ganglia to these muscle-nerves is, for our purpose, if it exist at all, numerically negligible.

(*c*) *Spinal Ganglion.*—Though in these muscle-nerves a large proportion of their small fibres even down to 2 μ diameter derive directly from the ventral spinal roots many fibres similarly small, even down to 2 μ diameter, remain still sound after severance of those roots. Conversely many degenerate after extirpation of the dorsal-root ganglia.

The spinal ganglia as a source for the small myelinate fibres were further examined by ablating the ganglia, in series of three, for their *proximal* parts only, so as to escape all risk whatever of touching the ventral roots. The degenerative periods allowed were 5½ and 6½ days, the search being for degeneration in the smallest fibres. The observed degeneration in the muscle-nerves affected many quite small fibres, some down even to 2 μ diameter. Ranson's (18)

(1914) fibres are, of course, non-myelinated; it is with myelinated only that our problem is concerned.

(*d*) *Dorsal Spinal Root.*—Finally, further observations were made in which the dorsal roots 8, 7 and 6 were cut within the spinal theca well proximal therefore to the root ganglion; and without exposure to view of the ventral roots, which were carefully avoided. Degenerative periods of 5, 7 and 10 days were allowed and the muscle-nerves then fixed in osmic acid. Examined by teazing, after mild dissociation, not a single degenerating myelinate fibre was ever found in any of the muscle-nerves from any of these experiments. This negatived the provenance of any of the myelinate fibres, small or large, of these muscle-nerves from intraspinal cells *via* the dorsal roots.

The question of the immediate source of the small myelinate fibres which remain sound in these muscle-nerves after ablation of the root ganglia received therefore a clear answer. *All of them, the smaller no less than the larger, derive immediately from the motor spinal roots.*

(*e*) *Source of the Small Efferents* (i) *from Small Ventral-root Fibres, and* (ii) *from Large Dichotomising Nerve-trunk Fibres respectively.*—Are these small efferent fibres of the muscle-nerves traceable merely to dichotomy of larger fibres in the course of the nerve-trunks, or are some of them in fact the small fibres of the ventral spinal-root continued distally into the muscles? From the motor-unit point of view the meaning of the small efferent fibre is greatly different in the two cases. In the former each such fibre represents only a fraction of a unit, in the latter each represents an entire unit.

In regard to the pre-existence of the small fibres as such in the spinal ventral roots, the 7th ventral root although containing fibres up to 20 μ diameter has more than 35 per cent. of its fibres below 8 μ diameter; and the 6th ventral root very similarly. Some fibres in these roots are just as in the muscle-nerves as small as 2·5 μ (paraffin sections). The ventral roots of the region could therefore provide the small fibres as such. Indeed, as regards that feature which all the muscle-nerves here dealt with yield to analysis, namely, a fibre-size constitution having two peaks of numerical predominance centred on two fibre sizes widely apart (Charts 1–4), the ventral roots which contribute to these nerves present the same feature (Charts 4 and 5). The normal afferent-efferent nerve of gastrocnemius has also the same feature (Plate 28, fig. 1). The subjoined analysis illustrates this (Table III). The motor root has its fairly discrete block of smaller fibres as has the peripheral motor-nerve. It is possible therefore for the block in the latter to be mainly derived from the former.

Table III.—The smaller of two divisions of normal *gast. med.* nerve (Plate 28, fig. 1).

Fibre diameter in divisions of ocul. microm. (1 division = 2·5 μ.)	Number of fibres.	Percentage of total of the fibres.
Less than 1 division	27	9
1 to 1·5 divisions	21	7
1·5 to 2 divisions	39	13
2 to 2·5 divisions	28	9
2 to 3 divisions	7	2
3 to 3·5 divisions	3	1
3·5 to 4 divisions	13	4
4 to 4·5 divisions	11	4
4·5 to 5 divisions	31	10
5 to 5·5 divisions	23	8
5·5 to 6 divisions	62	21
6 to 6·5 divisions	20	7
6·5 to 7 divisions	17	6

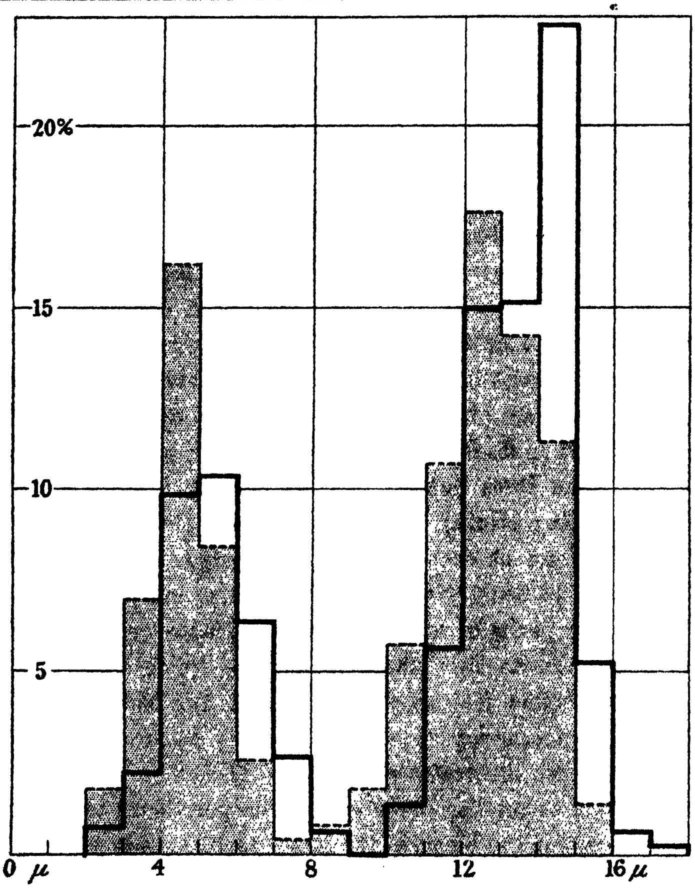


Chart 1.—Nerve-fibre diameters in μ plotted against numerical distribution in percentage of total efferent fibres in nerve of *gast. med.*, solid line (——), of *ext. l. dig.* broken line (- - -).

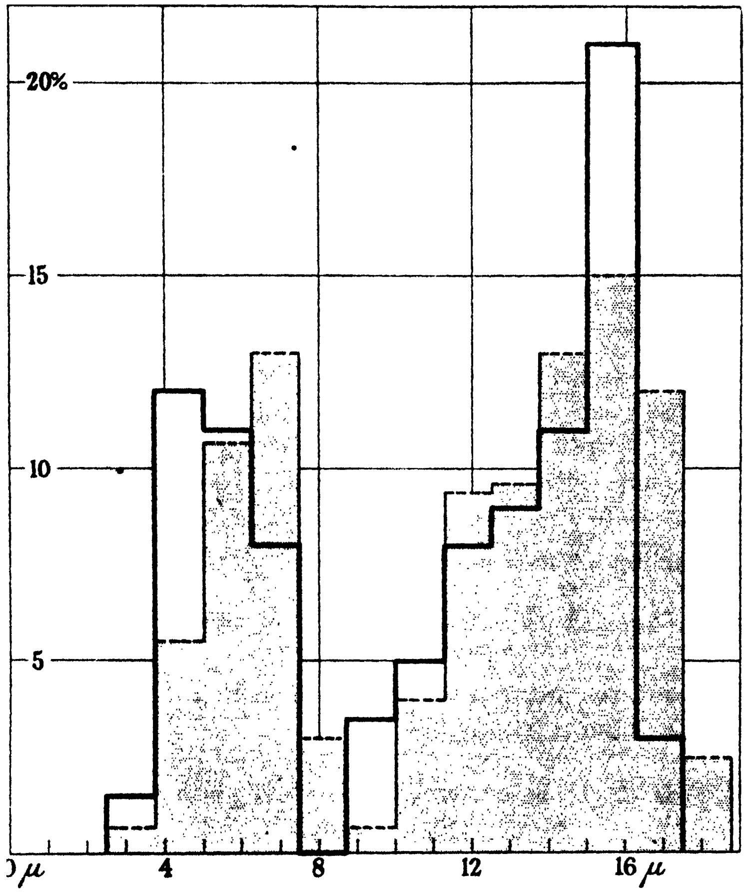


CHART 2.—As Chart 1, but for deafferented nerve of *biceps femoris* (——) and deafferented nerve of *semitend.* (- - -) (*cf.* Plate 28, fig. 2, Plate 29, fig. 9).

On the other hand it is to be remembered that the splitting of motor-fibres certainly decreases the average diameter of the motor-fibres as the nerve is traced peripherally. In studying the dichotomy we have found in every case the parent-fibre thicker than either of its daughter-fibres, though often only slightly so. Sometimes the twin daughter-fibres appear just equal one to the other in size, sometimes they are very unequal. This agrees with the observations by Cooper (11) (1927). She records, however, some parent-fibres smaller than any we have met. The difference may be related to our search being made not so near the muscle. Once only have we seen high up the nerve a splitting motor-fibre of less than 10 μ diameter; most have been of

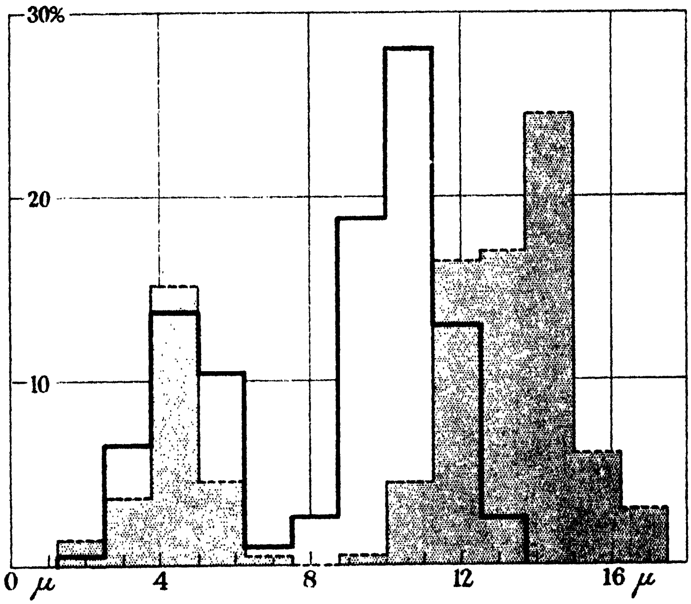


CHART 3.—As Chart 1, but for deafferented nerve of *soleus* (——), and deafferented nerve of *gast. lat.* (- - -).

14 μ or upwards, the preponderant size has been 16 μ. We have not met a daughter-fibre of less than 6 μ.

Table IV.—Samples of motor Nerve-fibres Splitting high up in their respective Muscle-nerves.

Parent-fibre diameter in μ.	Branch 1.		Branch 2.		Muscle-nerve.
	Diameter in μ.	Length to next node in μ.	Diameter in μ.	Length to next node in μ.	
22	16	1280	14	1160	*gast. med.*
17	15	910	15	930	,,
14	12	—	6	—	,,
11	9	—	7	—	,,
18	16	—	10	—	*semit.*
18	14	—	9	—	,,
15	12	—	11	—	,,
12	10	—	10	—	,,
17	16	1290	6	460	*semimemb.*
16	14	1240	8	810	,,
15·5	11·5	1190	11·5	1210	,,
14·5	11	960	11	950	,,
15	14	—	10	—	*n. abducens*

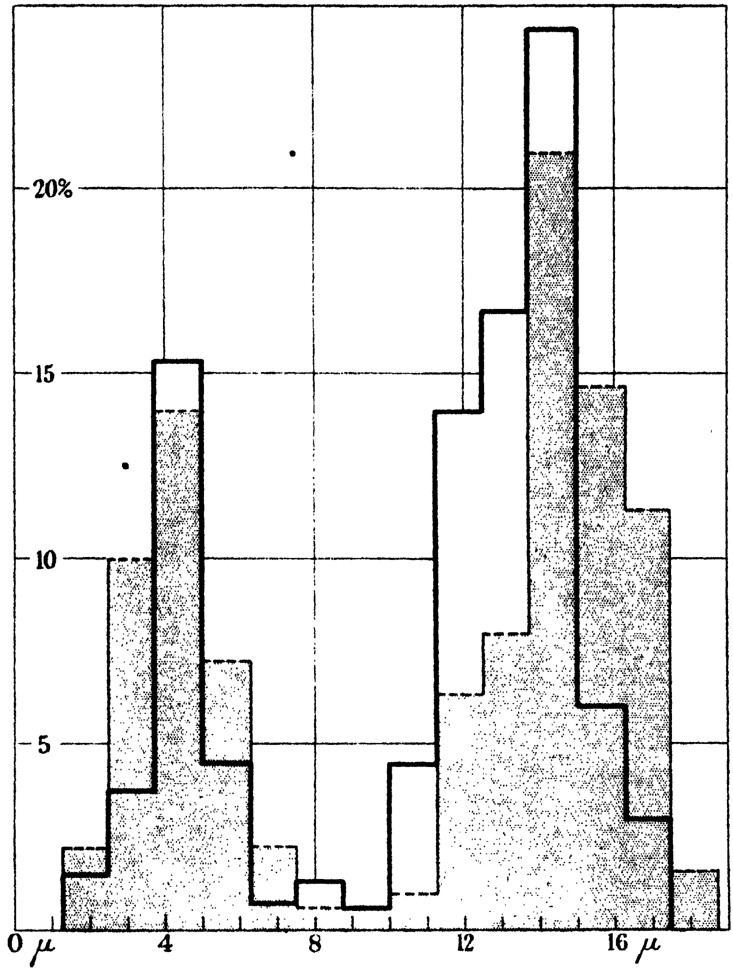


CHART 4.—As Chart 1, but for deafferented nerve of *gast. lat.* without *soleus* (——), and for 300 fibres of 7th ventral spinal root (- - -).

The percentages of fibre-sizes in the deafferented nerve of *gast. med.* at 50 mm. and at 8 mm. from the muscle are compared in Chart 6. At the more distal level, though the double peak is strikingly maintained, the coarser-fibre peak is reduced, the slender-fibre peak increased ; some of the largest fibres have as such disappeared. Fibres below 6 μ have relatively increased. There is a partial filling of the gap between two peaks, and the coarser-fibre peak has undergone a skew deviation toward the small-fibre side. The items of nerve-fibre calibre to-day present enhanced interest owing to the fundamental work of Erlanger and Gasser (13).

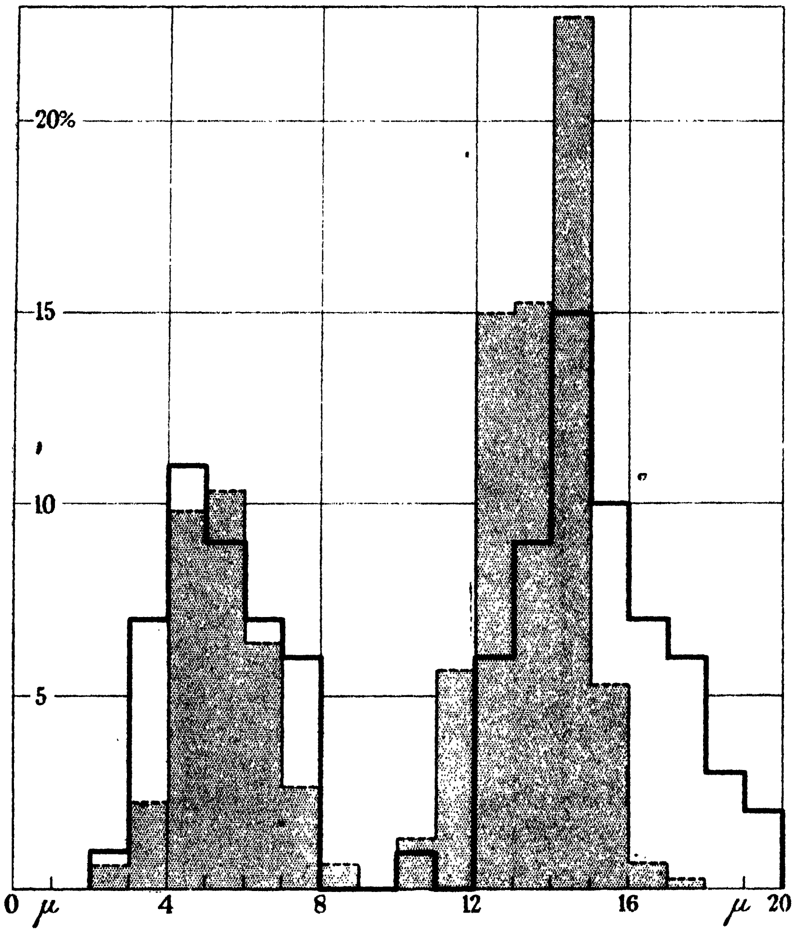


CHART 5.—As Chart 1, but for 100 fibres of 8th ventral spinal root (——), and for deafferented nerve of *gast. med.* (- - -).

To sum up, it seems clear that the smaller fibres present at distal levels in these deafferented muscle-nerves have, in not negligible proportion, arisen by the splitting of larger fibres within the nerve-trunks, especially distally in their course. But the number of 2 μ to 8 μ motor-fibres in these nerves is too great to have arisen chiefly in that way. Further, at long distances, *e.g.*, 60 mm. above the muscle their number is already quite large, *e.g.*, 33 to 34 per cent. of the total nerve, although there the rate of numerical increase as observed for splitting is too low for proximal dichotomy to explain the number present. Besides small fibres (2–8 μ) are already plentiful in the ventral root itself (Charts 4 and 5), and in that root as Dale's (6) countings show there

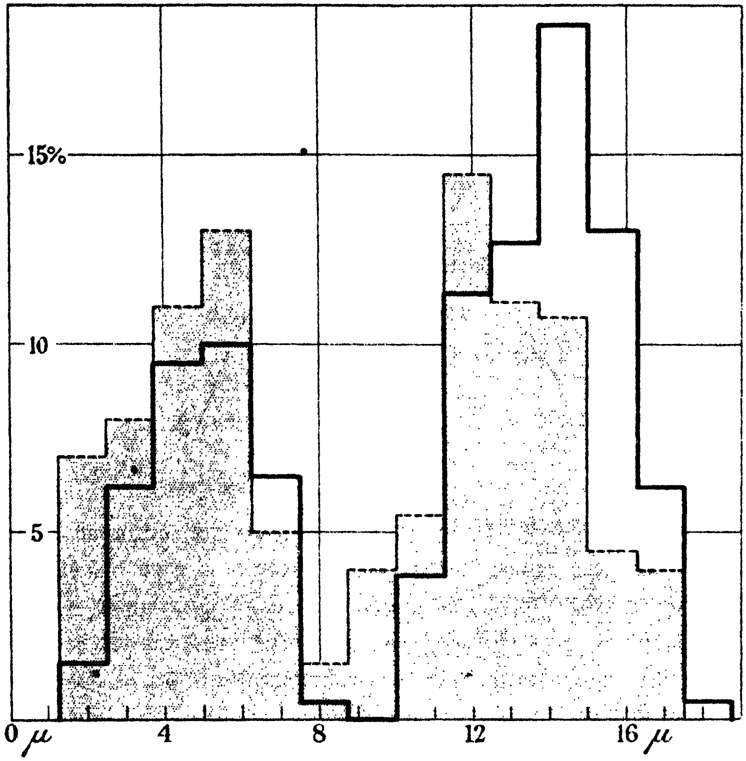


CHART 6.—As Chart 1, but for deafferented nerve of *gast. med.* 50 mm. from entering muscle (——), and 8 mm. from entering muscle (- - -).

is no multiplication of fibres. The observations argue that the small efferent fibres have for the most part emerged as such from the spinal motor root, and are in fact small fibres of that root destined for the skeletal muscles.

(4) *Incidental Observations on Afferent Fibres in the Muscle-nerves.*—A passing digression may be allowed on some incidental observations regarding the afferent fibres in these muscle-nerves.

As mentioned above, Dunn (1900) ascertained (frog) a numerical increase of fibres in *afferent* nerves in their distal direction. We have examined (cat) our type muscle-nerves after reducing them to pure afferents by destruction of all their motor fibres. The counts then taken show the number of afferent fibres close to the muscle to be distinctly greater than at distances further back.

Thus :—

—	Millimetres from muscle.	Number of afferent fibres.
Gast. med. (1)	about 50	240
	,, 8	264
Gast. med. (2)	,, 40	258
	,, 20	261
	,, 8	275
Gast. lat. soleus (3)	,, 60	451
	,, 40	448 (?)
	,, 10	472
	,, 5	499
Gast. med. (4)	a bundle before breaking up	131
	same after breaking up	146

In instances (2) and (3) the increase of afferent fibre-number is seen to rise in rate with nearness to the muscle, as was seen also with the efferent fibres. Multiplication is, however, in these nerves less in the afferent than in the motor-fibres, nor does the branching of the afferent fibres appear to begin so far proximal in the nerve-trunk. The actual demonstration of splitting afferent fibres in these nerves has, in spite of frequently repeated and lengthy search by the same methods which finally succeeded in the case of the splitting motor-fibres, proved almost fruitless. Only in one instance was a splitting afferent nerve-fibre found ; it was in a *soleus* nerve in which all motor-fibres were in advanced degeneration, fourteen days after section of 6th, 7th, 8th and 9th ventral roots. Among the sound fibres was a small parent of 5 μ splitting into two daughter-fibres of 5 μ and 3 μ. No other splitting afferent fibre was ever found, although many " demotored " muscle-nerves were searched.

The above instance amounts to a 5 μ myelinate fibre giving off in its course a 3 μ myelinated branch. With the increase in number of afferent nerve-fibres in the muscle-nerve as the muscle is approached goes increase in the small afferent nerve-fibres relatively to the larger. Thus, *gast. lat.* (including *soleus*) *n.*, completely demotored ; count of afferent fibres at 60 mm. from muscle, 451, of which 233 of 10 μ diameter or less, and 218 above 10 μ ; count of afferent fibres at 5 mm. from muscle, 499, of which 287 of 10 μ diameter or less, and 212 above 10 μ.

The *soleus n.* when deafferented shows a striking difference *v.s.* (fig. 4, Plate 28) from the other muscle-nerves similarly deafferented. On the other hand, when demotored, it presents no such obvious difference from them

(Fulton (5), Hay (20)). As regards heterogeneity of fibre-size, the muscle afferents are strikingly more heterogeneous than are the ordinary afferent nerves, *e.g.*, cutaneous. Chart 7 illustrates this, comparing the proximal end

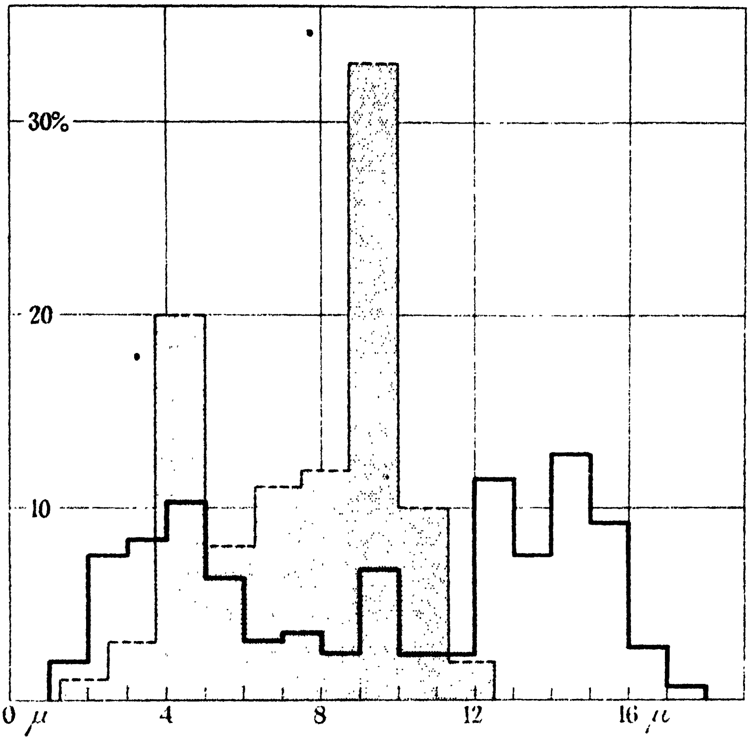


CHART 7.—As Chart 1, but for "demotored" nerve of *gast. med.* consisting of 242 afferent fibres, solid line (——), and for a dorsal digital nerve at its proximal end sampled by 200 fibres (- - -).

of a dorsal digital nerve with the proximal part of (demotored) *gast. med.* nerve. Nerve of *soleus* does not separate out from the *gast. lat. soleus* complex much before reaching the muscle; the afferent fibres in it are at largest about 13 μ, whereas those in the *gast.* nerves run to 18–19 μ. But in the digital nerve the fibres of 13 μ are relatively fewer than in *n.* of *soleus.*

The branching of the afferent nerve-fibre in the course of its approach to its muscular destination offers interest in regard to the degree of proprioceptive "local-sign."

(5) *Motor Nerve-fibre Calibre and Motor-unit Size.*—Diameter of motor-fibre obviously suggests itself as some clue toward the size of the motor-unit. A large nerve-fibre seems as judged by branching to embrace more muscle-fibres than does a small, the calibre of the nerve-fibre *cæteris paribus* bearing

relation to its amount of branching. With the motor-fibres observed to split in the nerve-trunks it is almost exclusively among the larger, *v.s.*, p. 340, that branching begins far proximal from the muscle, *i.e.*, the large fibre prepares early to form an extensive motor-unit.

When a nerve-fibre divides the sum of the cross-sectional areas of the daughter-fibres is commonly found to be slightly greater than the cross-sectional area of the parent-fibre. Thus, taking the samples furnished in Table III, *v.s.*, the three groups there give a ratio of sectional area of combined daughter-fibres to the cross-area of parent as follows: 1·11, 1·08 and 1·05. The diameters measured were those of the total fibre, *i.e.*, including myelin sheath, but in the cross-area of the total fibre the ratio of area of axis cylinder to area of myelin sheath is very closely 1 : 1 (Donaldson and Hoke (14) (1905)). The dichotomy-ratio mentioned above may therefore be applied to the axis cylinders themselves. If the parent-to-daughter ratio hold for the further splittings and the final branches, each of which ends on a separate muscle-fibre, be uniformly fine, the cross-sectional area of the parent-fibre gives an indication of the proportional size of the motor-unit.

A nerve-fibre of 20 μ which divides when still distant from its muscle into daughter-fibres relatively large, themselves in turn to divide into further dichotomising branches, will annex more muscle-fibres and compose a more extensive motor-unit than does a small fibre which, issuing from the spinal cord with a diameter of 5 μ, does not before reaching its muscle branch at all. Judged on these grounds the aggregate of motor-units of a given muscle will not consist of individuals very uniformly sized. That the calculation of the average tension-value of a motor-unit, obtained by dividing the total tension by the total nerve-fibres, will give but a rough estimate of value of the prevalent unit, is clear from the widespread about their mean revealed by the nerve-fibre diameters, and still more as reckoned by their cross-sectional areas. Further, no account is thus taken of the double-peak fibre-size distribution and the distinct grouping into small and large fibres. Since the motor-plate is, in skeletal muscle, the only known ending of myelinated ventral-root fibres, it is provisionally assumed here that even these smallest myelinate fibres supplied from ventral roots are also "motor." Table V gives an analysis of the results of a differential count, with 1 μ steps, of the total efferent fibres of *gast. med.* nerve. The total cross-sectional area of each group of fibres is calculated; column 6 gives the percentage cross-sectional area of each group (see Chart 8).

Table V.—*Gast. med. n.* Experiment 1 of Table VI, p. 348.

Diameter of fibre.	Sectional area of single fibre.	Total fibres of group.	Percentage of fibres.	Percentage multiplied by area of single fibre.	Area of each group as percentage of total area.
μ	× π/4			× π/4	
2–3	6¼	3	0·7	4·4	0·03
3–4	12¼	11	2·25	27·6	0·2
4–5	20¼	48	9·9	200·5	1·5
5–6	30¼	50	10·3	311·6	2·4
6–7	42¼	28	6·35	268·3	2·0
7–8	56¼	12	2·65	149·1	1·1
8–9	72¼	3	0·7	50·6	0·4
9–10	90¼	0	0	0	0
10–11	110¼	6	1·35	148·9	1·1
11–12	132¼	24	5·75	759·4	5·7
12–13	156¼	60	15	2343·7	17·5
13–14	182¼	61	15·25	2779·3	20·8
14–15	210¼	97	22·8	4793·7	35·9
15–16	240¼	23	5·2	1249·5	9·4
16–17	272¼	3	0·7	190·6	1·4
17–18	306¼	2	0·25	76·6	0·6

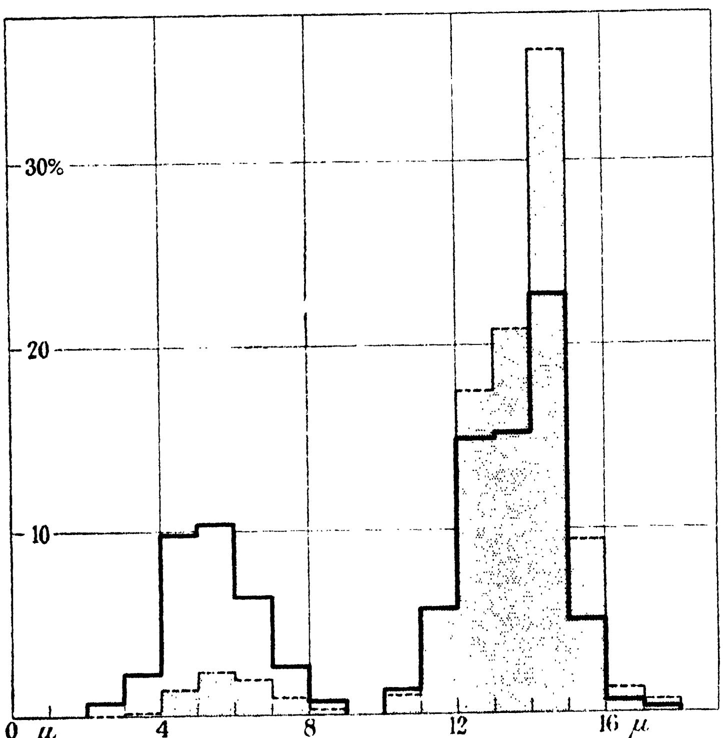


CHART 8.—As Chart 1, but with cross-section area (see Table V, column 6) of fibre-size groups in percentage of total axis-cylinder area expressed as ordinates: de-afferented nerve of *gast. med.*

It will be seen that the group of large fibres, *i.e.*, above 10 μ diameter, although only 66·3 per cent. in point of numbers, forms 92·4 per cent. of the total cross-sectional area; it probably supplies a somewhat similar proportion of the muscle-fibres. By dividing the percentage cross-sectional area of any group by the number of fibres in that group a figure is obtained which gives some indication of the percentage of the total tension of the muscle which is developed by a fibre of that group, and so provides a rough estimate of the contraction-tension value of the motor-unit of the fibre.

(6) *Contraction-tension of the Average Motor-unit of the Muscles Examined.*

In the accompanying Table VI experiment 2 is the only one where excision of a root ganglion was found on microscopic examination to be incomplete. The distal part of ganglion 9 had escaped removal. But our uniform experience

Table VI. (*cf.* Chart 9).

Experiment.	Muscle.	Weight of animal in kilograms.	Spinal ganglia extirpation.	Days of degeneration.	Contraction tension.		Number of motor-fibres.	Contraction tension average per motor-unit.	
					Maximum twitch.	Maximum tetanus.		Twitch.	Tetanus.
1	*gast. med.*	3·6	6, 7, 8, 9	79		14800	431		34·3
2	,,	3·5	6, 7, 8 (9)	75		13900	437		31·8
3	,,	3·3	5, 6, 7, 8	60		12700	388		32·7
4	,,	3·4	6, 7, 8	52		13900	459		30·3
5	,,	3.4	6, 7, 8	45		15800	448		35·2
6	,,	2·15	6, 7, 8	82		7290	307		23·6
7	,,	2·1	6, 7, 8, 9	48	2500	9080	393	6·4	23·1
8	*soleus*	3·4	6, 7, 8	45		1940	198		9·8
9	,,	2·5	6, 7, 8	52		1780	174		10·2
10	,,	2·1	6, 7, 8, 9	48	580	2230	233	2·48	9·57
11	*semitend.*	3·5	6, 7, 8, 9	77		3800	636		5·97
12	,,	3·4	6, 7, 8	52		4100	655		6·2
13	,,	3·3	5, 6, 7, 8	60		3700	632		5·86
14	,,	3·4	6, 7, 8	45		3800	599		6·3
15	,,	2·5	6, 7, 8	52		2700	436		6·2
16	,,	3·6	6, 7, 8, 9	79		4500	667		6·7
17	,,	2·15	6, 7, 8	82		3400	606		5·6
18	,,	1·7	5, 6, 7, 8, 9	55		1690	289		5·8
19	,,	2	5, 6, 7, 8	44		1990	362		5·5
20	,,	1·8	5, 6, 7, 8	48		720	126		5·7
21	,,	2·1	6, 7, 8, 9	48	1020	3310	549	1·8	6·02
22	*ext. l. dig.*	3·3	5, 6, 7, 8	60		2500	297		8·4
23	,,	3·4	6, 7, 8	45		2950	317		9·3
24	,,	2·1	6, 7, 8, 9	48	710	2010	247	2·8	8·1
25	*crureus*	3·3	5, 6, 7, 8	60	690	2600	256	2·7	10·2

has been that root 9 gives no contribution to *gast. med.* or *semit.*, which were the muscles investigated; the experiment is therefore not rejected.

The muscular contractions observed were usually below, in some instances much below, the full normal strength. The deficit was probably due entirely to partial injury of a motor-root or of motor-roots at the deganglionation operation. The evidence from some of the experiments indicates clearly that desensitising (deafferenting) the muscle does not *per se* impair its contractile power. In our earlier experiments the maximal contraction of the deafferented muscle was as a matter of routine, compared with that of the fellow muscle of the unoperated side.

Table VII.

Muscle.	Period.	Deafferented.	Normal.
	days	k.	k.
Gastroc. med.	79	14·8	15·4
,,	75	13·9	14·3
,,	45	15·8	16·4
Semitend.	77	3·8	3·9
,,	79	4·5	4·9
,,	18	1·96	2·04
,,	52	4·1	4·25

The approximate or complete equality of contractions on the two sides, although the escape from incidental damage to the ventral roots was exceptional, does by its occurrence rule out the suspicion that deafferentation *per se* impairs the motor power.

In Table VI, looking at the figures found, we bear in mind that, as shown in previous sections of this paper, the individual motor-units composing a given muscle must be expected to depart rather widely from uniformity in size. It has been shown above (Charts 1–6) that the numerical distribution of motor nerve-fibre sizes does not, as might have been expected, fall on a simple probability curve about a single mean, but offers two predominant sizes rather widely apart. The evidence argues that there will be a group of large units and a group of smaller. The mean value of the average motor-unit does not make explicit this division into two groups.

The largest average motor-unit tension was yielded by *gast. med.* and consistently so; *semit.* consistently gave the smallest (Chart 9). Intermediate but nearer to *semit.* were the values found for *ext. l. dig.*, *soleus* and *crureus*, this last resting on a single experiment. The observations on *tibialis anticus* were imperfect; they gave a value close to that of *ext. l. dig.* The

influence of fibre-splitting, lowering the estimate of motor-unit value, would be greatest for *ext. l. dig.*, owing to the shortness of its available length of nerve ; and fibre-analysis (Chart 1) of its nerve suggests influence from proximal fibre-splitting. Probably therefore the average motor-unit tension-value for *ext. l. dig.* is somewhat understated. That obtained for *semit.* is still lower, although the nerve fibre-counts were made well distant from the muscle.

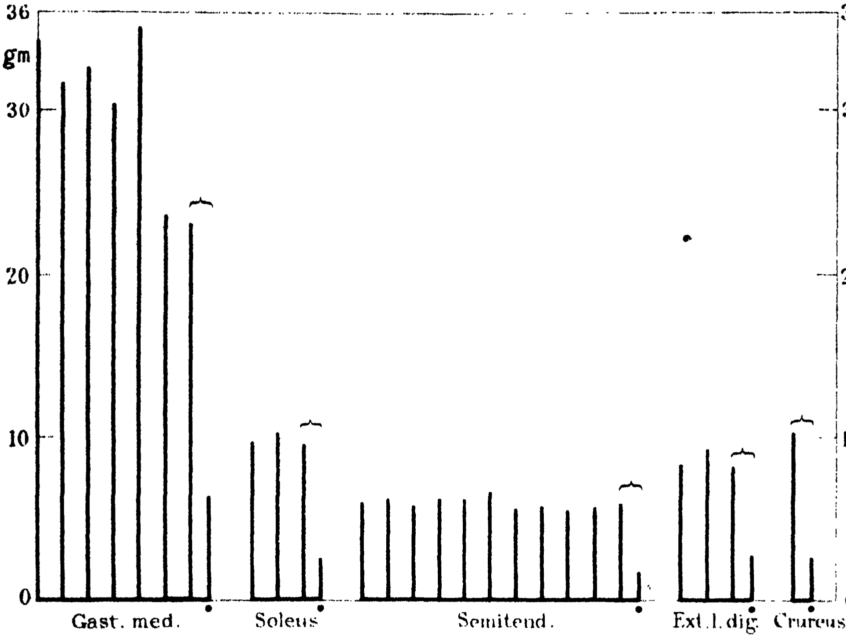


CHART 9.—Columns 6 and 7 of Table VI. with contraction-values expressed in grm.-tension as verticals ; a subjoined dot indicates a twitch-contraction ; brackets conjoin tetanic and twitch values from the same experiment.

Semit. consists of two fleshy parts connected, by intervening fibrous tissue, in series not in parallel for their line of pull. Isometric contraction tension will therefore assess imperfectly the contractile value of the muscle (Cooper, 1929 (11)). Its motor-unit will be judged at a disadvantage. That unit in number of muscle-fibres may average larger than from the tension-value appears. Its nerve is double (*cf.* Plate 29, fig. 9), and both divisions are not inferior in motor nerve-fibre size (Chart 2) to the other muscle-nerves examined and are considerably superior to *soleus* (Plate 28, fig. 4, and Chart 3) nerve.

Soleus would seem, arguing from its nerve-fibres, the least heterogeneous of the muscles examined. Its motor nerve-fibres, although exhibiting the double peak fibre-size distribution (Chart 3) have their extremes of fibre-size less apart

than have the other muscles, and 28 per cent. of them are almost exactly of a size (*cf.* Chart 3). Its small-fibre block resembles that of other muscles; its block of large fibres centres about 11 μ instead of, as in the other muscles, 15 μ. Its average nerve-fibre is therefore smaller than that of *gast. med.* and so likewise is its motor-unit.

The mean (7 experiments) arrived at for *gast. med.* is 30·1 gm. With this may be compared an estimate obtainable from an earlier published (19) count of afferent and total fibres in *gast. med.* nerves (cat). Of these nerves that of the normal side totalled 946 fibres, that of the operated, *i.e.*, with all motor-fibres removed, 375 fibres. The weight of the cat was not stated, but the numbers from both nerves show it to have been large, somewhat larger than any in the present research. Assessing its maximal contraction therefore at 17,000 gm. (the largest maximum contraction met in normal *gast. med.* in the course of the present experiments was 16,400 gm. in experiment 5, a 3·4 k. cat), and assuming right and left nerves to contain the same number of fibres, the estimated contraction-tension "average" for the motor-unit would be 29·7 gm., a near approach to the above actually observed and measured 30·1 gm.

We may now revert to the inference drawn above from nerve-fibre calibre that the motor-units of a muscle fall into two main groups owing to predominance of two widely different sizes. This complexity can presumably be met by estimating the tension-value of the particular motor-unit by the procedure of dividing the tension developed by the muscle by the ratio of the cross-area of the particular unit's nerve-fibre to the aggregate cross-area of the motor nerve-fibres of the muscle.

The contraction-tension of the motor-unit found for quite small individuals was smaller than for large. Thus :—

Cat 3·6 k., *gast. med.*	average motor-unit	34·3 gm.
,, 2·1 k., *gast. med.*	,,	23·1 ,,
,, 3·6 k., *semit.*	,,	6·7 ,,
,, 2·1 k., *semit.*	,,	6·02 ,,
,, 3·4 k., *soleus*	,,	9·8 ,,
,, 2·1 k., *soleus*	,,	9·5 ,,
,, 3·4 k., *ext. l.d.*	,,	9·3 ,,
,, 2·1 k., *ext. l.d.*	,,	8·1 ,,

The smaller cats were also the younger.

II. *Tension-Value of Sample Individual Motor-Units.*

The values obtained for the average motor-unit in the foregoing experiments are quantities evidently open to examination by a myograph. For postural reflexes of *soleus* Denny-Brown (15) (1929) was able to obtain myographic records of single motor-unit responses. With a more sensitive frictionless (4) (knife-edge) myograph (1 gm. tension producing 1 mm. deflection) an accurate tension-evaluation has been possible. Since all surrounding muscles and fascia would interfere greatly with such weak responses, it was necessary to isolate the muscle completely except for the nerve and blood supply.

The muscular contractions were evoked reflexly (tendon-jerks) because grading is more easy to attain in that way than by direct stimulation of the motor-nerve. Fig. 10 (Plate 30) is taken from an experiment in which *soleus* tendon-reflexes were elicited by taps of an insulated rod on the standard fixating the muscle. When using a sensitive myograph this method is preferable to tapping the actual tendon, because the mechanical disturbance by the tap is less long-lasting and its earlier subsidence leaves the myographic record freer.

By alteration of the strength of the tap it is possible to obtain a grading of response in normal tendon-reflexes, but it is quite difficult to adjust the stimulus so as to produce a reflex-response confined to very few motor-units. However, when the ventral roots (and in the experiment of fig. 10 also some of the dorsal roots, see legend) supplying the muscle are largely cut down, so that the maximum response itself can involve but few units, a simple grading of response can be obtained and the gradation is seen to be steplike.

Below are given in order of magnitude the tension-values in grams of each reflex twitch (the repetitive after-effects in two further instances included in the figure are discussed later) of fig. 10 :—0, 0, 2·5, 2·5, 4, 6·5, 6·5, 6·5, 6·5, 7, 8·5, 10·5. The concurrent record of the string-galvanometer (Ag-AgCl leads in lateral border of muscle) shows absence of electrical response when the myograph registers absence of reflex contraction. On both occasions when 2·5 gm. tension is produced there is a simple diphasic electrical response, while a clearly double action-current is shown in the 4 gm. twitch (second response on top line). Moreover, the electrical response in this case shows that the first action-current is the same as in the 2·5 gm. reflex, while the second is possibly identical with that involved in the repetitive discharge following two of the twitches. In all of the larger twitches close inspection shows electrical responses compounded of more or less asynchronous single action-currents.

There seems no reason to doubt that the 2·5 gm. responses are twitches of

single motor-units, and the 4 gm. responses of 2 units. Continuing the series it seems likely that 6·5–7 gm. is given by 3 units, 8·5 gm. by 4 units, and 10·5 gm. by 5 units. In this series 2·5 gm. is the greatest tension of a single unit and 1·5 gm. is the smallest. Further observations during the experiment accorded perfectly with these values; in all cases 1·5 or 2·5 gm. was the tension produced by a single unit.

In fig. 10 there are also examples of tetanic responses in a single unit accompanied by simple electrical diphasic responses of similar rhythm in the electrical record exactly corresponding with the undulations in the myographic record. Owing to the gradual slowing of the rhythm a steady plateau height is not maintained; when, however, the same tension is developed in two successive responses it can be assumed that this tension corresponds with the tetanic plateau at the particular rhythm of those responses. Tension-values in fig. 10 are 8·7 gm. at 10 per second, and 8 gm. at 9·5 per second.

Another record in the same experiment shows 7·2 gm. at 13·7 per second for another unit. The rhythm of stimulus being so low the maximum tetanic tension is not reached in these instances. An approximate calculation made from data on maximum stimulation of the muscle at various rhythms (Cooper and Eccles (23)) gives for the greatest tetanic tension 10·4 and 10·0 gm. (average 10·2 gm.) for the first unit and 8·0 gm. for the second unit. The tetanic tension-values of the two units agree well with the value obtained by the "averaging" method (*v.s.*, § I), and the tension-values of units show a spread corresponding with the spread observed for (*v.s.*, Chart 3) the group of larger efferent fibres of *soleus* nerve. The low frequency (9–10 per second) of the reflex tetani observed in the individual unit agrees with the observations of Denny-Brown (16) and of Adrian and Bronk (17).

2. The Number of Motor-units composing the Normal Muscles.

In most of the experiments a fraction of the muscle's motor nerve-fibres had suffered incidental damage at the ventral spinal root during performance of the operation on the ganglia. Sometimes as shown by subsequent myographic comparison of the operated with the unoperated side the amount of such injury was very small. In those cases the count of the efferent nerve-fibres gave a close approximation to the numerical aggregate in motor-units of the normal muscle. Thus estimated normal *gast. med.* consists of about 430 motor-units; *semit.* of about 640.

Soleus nerve in a cat of 2·1 k. contained, counted as far proximal as practicable, 233 efferent fibres; the muscle's contraction was not compared myographically with that of the unoperated side, so lacks that test of its degree of approach to normal size. It gave 2230 gm., which cannot have fallen far short of full normal size, since 2500 gm. is in our experience the average for a 2·5 k. cat. The average motor-unit contraction-tension found for *soleus* was (*v.s.*) 9·8 gm. The full normal muscle should thus contain about 250 motor-units.

Data for comparison with this are obtainable from the rabbit (Hay, 20). There the *soleus*, weighing 1·5 gm., instead of more than 3 grms. as here, was estimated, by subtracting the number of fibres in its demotored nerve from that in the normal fellow nerve, to receive 176 motor nerve-fibres.

Ext. l. d. received, in a 3·4 k. cat, 317 efferent fibres. The full normal value of the maximum tetanus of this muscle in a cat of that size can be put at 3250 gm.; in the 317-fibre cat it was 2950 gm. The finding for the average motor-unit is 8·6 gm. (*v.s.*), probably, for reasons above given, p. 350, a little too small. The normal muscle should therefore contain close on 330 motor-units.

3. Summary.

1. In each of four typical limb muscles the contraction-tension for their "average" motor-unit was examined by dividing the tension of the isometric maximal contraction into the number of nerve-fibres found in the muscle-nerve after removal of the dorsal root ganglia. The figures obtained were: *gast. med.*, 30·1 gm. (average of 7 experiments); *soleus*, 9·9 gm. (average of 3 experiments); *semit.*, 5·5 gm. (average of 11 experiments); *ext. l. d.*, 8·6 gm. (average of 3 experiments). *Crureus*, examined in a single experiment, gave 10·2 gm. In these values complete tetanic contraction is understood.

2. Of the motor nerve-fibres supplying the muscle some begin to dichotomise in the nerve-trunk several centimetres before reaching the muscle. This occurs increasingly frequently with nearer approach to the muscle. The nerve-fibre counts employed for enumerating the motor-units were therefore made as far proximal, *i.e.*, toward the spinal cord, as practicable. Under-valuation of the size of the motor-unit was thus considerably, perhaps completely, avoided, but least so for *ext. l. dig.*

3. In all the muscle-nerves examined, analysis of the motor fibre-sizes displays two peaks of numerical preponderance, centred on two fibre-sizes rather widely apart, *e.g.*, *gast. med.* 15 μ and 6 μ (measured in paraffin specimens, *i.e.*, slightly shrunk).

4. Of the group of small efferent myelinate fibres entering the muscle many exist as such several centimetres proximal to the muscle; it is concluded that these wholly or in large majority emerge as small fibres of approximately that calibre from the spinal cord *via* the ventral roots of the limb region. With approach to the muscle a number of additional myelinate fibres appear which are traceable as branches of larger fibres dichotomising higher up the nerve; these add themselves in increasing rate distally, and some of them swell considerably the group of small efferent myelinate fibres near to and entering the muscle.

5. The afferent nerve-fibres of the muscle-nerves examined exhibit increase in number, as followed distally in the nerve-trunk before it reaches the muscle.

6. The wide range of diameter, and therefore of cross-area, of the individual efferent fibres supplied by the ventral spinal root to each of the several muscles argues that the constituent motor-units of each muscle differ much one from another in size. This has to be remembered in regard to the "averages" observed.

7. Isolated individual motor-units, obtained reflexly as samples from *soleus*, gave values of twitch and tetanus in good agreement with the results for that muscle in the series of "averaging" experiments.

8. The observations arrive at approximate estimates of the number of motor-units composing the type-muscles examined, *e.g.*, *gast. med.*, 430; *ext. l. dig.*, 330; *soleus*, 250; *semit.*, 630.

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EXPLANATION OF PLATES.

Plate 28.

Fig. 1.—The smaller of the two divisions of a normal *gast. med.* n., at 45 mm. from muscle, × 350 This smaller division contains 302 myelinate fibres.

Fig. 2.—Portion of nerve of *biceps fem.*, all afferent fibres removed; ventral spinal-root fibres alone remaining, × 320; myelinate fibres from 2 μ–18 μ (*cf.* Chart 2).

Fig. 3.—From nerve of *semit.* about 35 mm. above entry into muscle; all afferent fibres removed; a large motor-fibre divides. × 425.

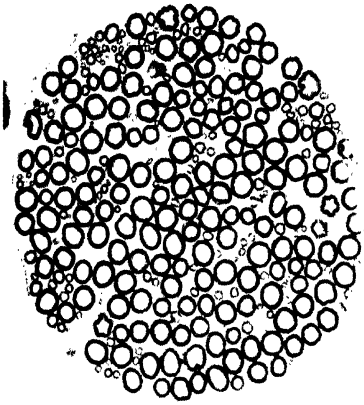
Fig. 4.—Nerve of *soleus* and of an accompanying branch of nerve of *gast. lat.* All afferent fibres removed. *Soleus* n. contains 174 fibres and gave a tetanic contraction of muscle of 1780 gm. The fibre diams. of *soleus* nerve average smaller than of *gastroc.* (*cf.* chart 3). × 128. Experiment 9 of Table VI, p. 348. For the converse of this figure, namely the afferent fibres after removal of all motor fibres instead of as here, the motors freed from all afferents, see Hay's figures VIII and X (20), reproduced by Fulton, p. 393 (5).

Figs. 5 and 6.—Splitting motor nerve-fibres in nerve trunk of *gast. med.* about 50 mm. above muscle; all afferents removed. Fig. 5, × 255; fig. 6, × 200.

Fig. 7.—Splitting motor nerve-fibre in nerve of *soleus* about 25 mm. above entry into muscle. The fibre is degenerating; the 6th, 7th and 8th ventral spinal roots having been cut 6½ days before; the dorsal roots and ganglia were untouched. × 300.

Plate 29.

Fig. 8.—*Gast. med.*; maximum contractions; isometric registration (torsion-wire on knife-edge bearing, with mirror projection. × 650); tetanus 9080 gm., twitch 2500 gm. Inset, the cross-section of the entire muscle-nerve, × 115; all afferent fibres removed by 48 days' degeneration; ventral root-fibres 393 in number. Average motor-unit, tetanic value 23·1 gm., twitch value 6·4 gm. Absence of all motor-fibres from a considerable patch in the cross-area of the nerve-trunk indicates that in the operation of removal of the ganglia one of the *ventral* roots had been partly trespassed on. Tuning-fork record, 1 d.v. = 0·01 second. Experiment 7 of Table VI, p. 348.



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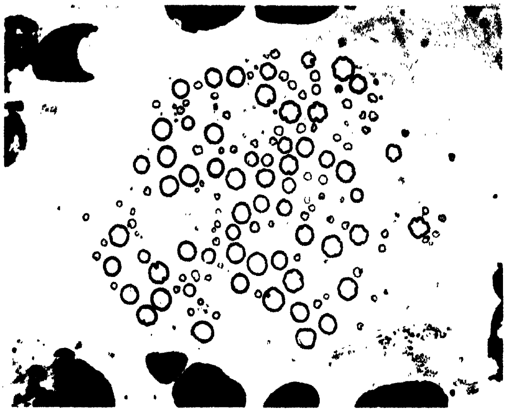

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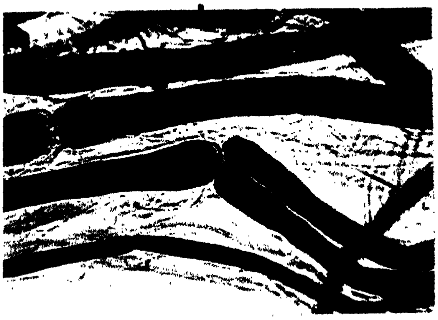

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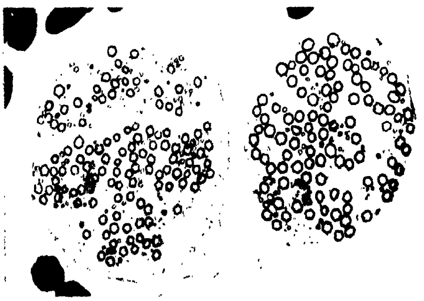

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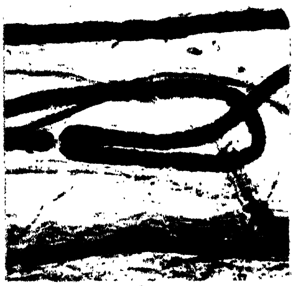

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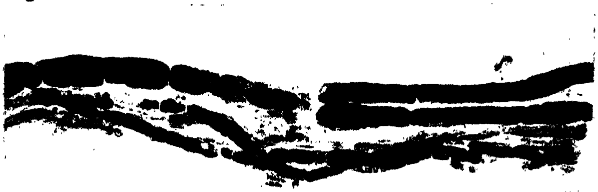

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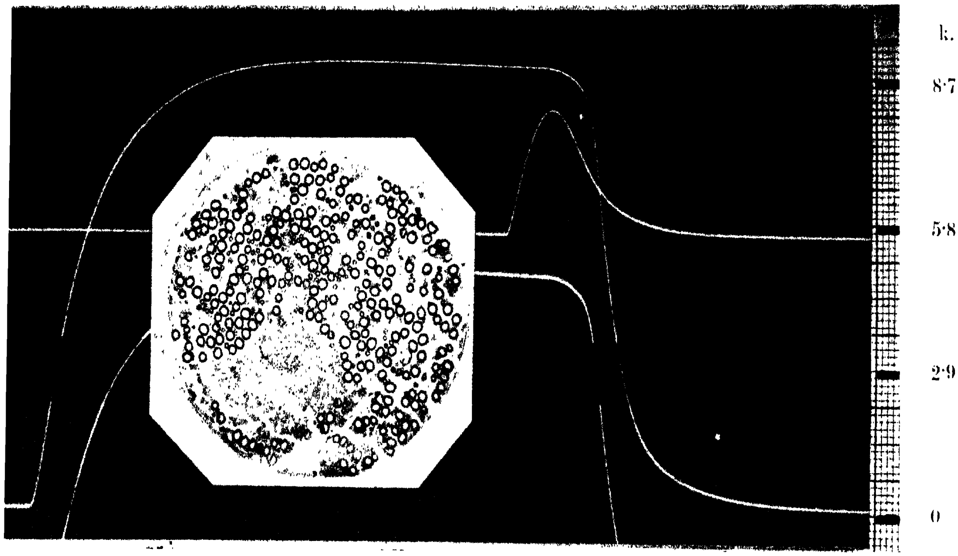

Fig. 8.

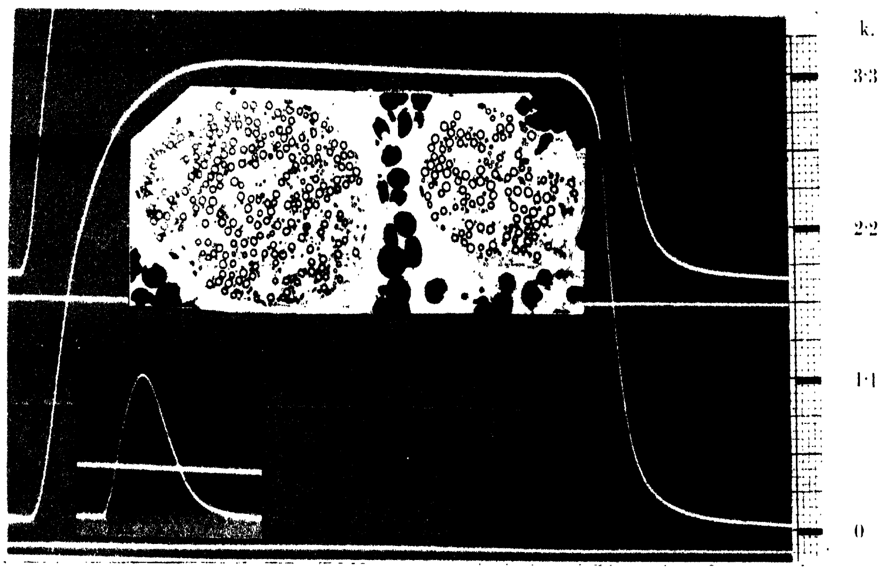

Fig. 9.

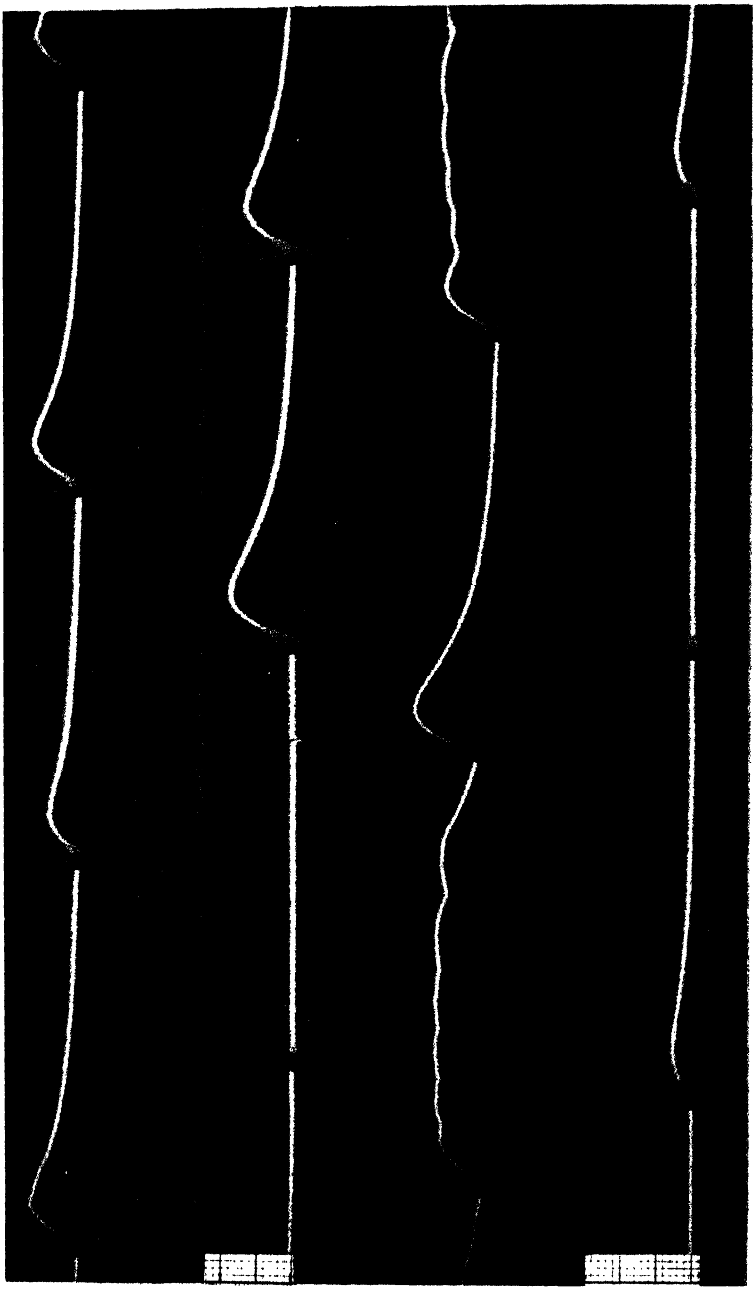

Fig. 10.

FIG. 9.—*Semitend.* Same animal and registration as in fig. 8. Maximum tetanus 3310 gm.; maximum twitch 1020 gm. Inset, cross-section of the entire muscle-nerve, consisting as usual of two divisions, × 90; all afferent fibres removed by degeneration; ventral root-fibres 549. Average motor-unit, tetanus value 6·02 gm., twitch value 1·8 gm. Time as in fig. 8. Experiment 21 of Table VI, p. 348.

PLATE 30.

FIG. 10.—*Soleus* muscle; decerebrate preparation; dorsal roots 6th and 8th and ventral root 7th, completely severed; also most of ventral root 8th. Reflex excitation by tapping standard fixating the muscle; mechanical effect of tap appears on the record as a rapidly damped oscillation. Next below the myograph record is record of string galvanometer; below that is the tuning-fork record, 1 d.v. = 0·01 second. Tension scale inset. 1 mm. ordinate = 1 gm. myograph tension. Further description in text, p. 352.

578 . 63 : 677 . 1

The Chemical Sectioning of Plant Fibres.

By M. A. EL KELANEY and G. O. SEARLE.

(Linen Industry Research Association.)

(Communicated by Sir Robert Robertson, F.R.S.—Received March 6, 1930.)

[PLATES 31, 32.]

The cellulose fibre has been investigated frequently in recent years, from both the chemical and physical points of view. Special attention has been paid to the minute details of visible structure, not only in connection with the problems of cellulose molecule arrangement but also as they may have a bearing on the technical problems of the textile industries. Summing our knowledge briefly, plant fibres have been shown to have a concentric structure of spirally disposed cellulose aggregates, with both crystalline and colloidal properties. Balls (1) in 1919 was able to link up the concentric structure of the cotton hair with the simple factor of a daily increment of growth. Reimers (2) in 1921 gave a useful summary of work on cellulose fibres, with special reference to the spiral arrangement of the fibre components, whilst Nodder (3) in 1924 brought this matter more up to date and extended our knowledge in several directions.

The present brief communication records another phenomenon, which

apparently has escaped notice before; namely, a transverse lamellation with a tendency under certain conditions for fibres to segment into thin sections perfectly transverse to the longitudinal fibre axis. This phenomenon was first described and illustrated by Searle (4) in a paper on chemically tendered flax fibres, but little attention was given to it at the time, as it appeared to have occurred more by accident than by design.

Recently, however, in a search for alternative methods of distinguishing between the many kinds of fibre received for routine identification, the possibility of controlling the section forming proclivities of a cellulose fibre were examined with greater care, as it was recognised that the appearance of the transverse section is a valuable diagnostic character. From a practical point of view the results exceeded expectation. From the theoretical point of view also they appear to be of considerable interest, but except in a quite tentative way this side of the subject will be left to others more competent to deal with it.

For brevity's sake we may refer to fibre sections formed by this method as "natural sections," in contradistinction to sections formed artificially by the use of a razor or knife. The original production of natural sections occurred on applying slight pressure to a preparation of flax fibres in a solution of caustic soda. The fibre was from a Courtrai flax, slightly tendered by storage for 20 years; the tendering was presumably due to a slow oxidation. As pressure was applied thin segments were seen to flake off the end of a fibre, fall over flat and thus appear as transverse sections of the fibre. Throughout our experiments with every kind of fibre the action is the same. The fibre on being tendered chemically and mounted in a caustic soda solution develops a very large number of fissures transverse to the fibre axis. The condition of the tendered fibre is analogous to a rouleau of coins lightly cemented face to face; a suitable blow can break off a segment and thus produces a transverse section. Normally, however, pressure applied to such a structure, especially when of the soft consistency of a wet mercerised fibre, results in a breakdown, transversely and longitudinally, into formless fragments.

In the first attempts to control the breakdown, flax and other bast fibres were soaked overnight in 1 per cent. sulphuric acid, dried in an oven at 96° C. until thoroughly tendered, mounted in an 11 per cent. solution of caustic soda and gently pressed. It was found that when the pressure was applied as a series of sharp taps on the cover-glass, so that a wave action was set up in the mountant, some of the fibres broke into segmental pieces, approximately 10 μ or so thick. The result was the appearance of many fibre sections

floating in the solution. All the sections were of single fibres and being swollen considerably by the caustic soda were rather abnormal in appearance for diagnostic purposes. When the tendered fibre was mounted in water or in very dilute caustic soda, this transverse lamellation did not take place.

Further experiments showed that the use of stronger acid, with a consequent more complete tendering of the fibre, caused not single fibres but whole fibre bundles to segment, so that more or less perfect sections of the fibre bundle were readily obtainable.

So far, the most successful method is based on the use of 10 per cent. sulphuric acid. A small quantity of fibre is boiled in the acid for a few minutes, roughly dried between filter papers without washing, and heated in an oven at about 60°—70° C. for, say, half an hour. The fibre should be taken from the oven as soon as it has become a deep brown to almost black. Small portions are mounted direct into caustic soda solution of 10-15 per cent. or alternatively they can be ground up gently in a mortar or by rubbing in paper between the fingers before mounting. A cover-glass is placed in position, excess caustic soda is mopped off and the cover is then tapped sharply and repeatedly with the tip of a scalpel until the fragments of fibre begin to disintegrate. Pulverising the tendered fibre before putting into caustic soda is found to ensure good sections of the more difficult subjects such as pineapple and *Phormium tenax* fibre, but is unnecessary with the larger fibres.

When using the dilute acid method it is necessary to stain the tendered fibre before mounting and a short boil in a solution of 0·5 per cent. congo red in 1 per cent. caustic soda will effect this satisfactorily. By the use of stronger acid, however, the fibres are partly charred and the sections when formed are a light brown colour very suitable for visual and photographic observation. The use of the stronger acid results in less swelling occurring in the caustic soda, so that the appearance of the sections is in every way more normal.

As it stands the technique appears somewhat crude; that it is reasonably successful for the object in view, namely to obtain sections of various plant fibres as quickly and simply as possible, can be seen from the illustrations accompanying this paper. Possibly the method might be standardised, if necessary, to give sections comparable in degree of swelling. As regards the mere production of sections we have been successful when using strengths of acid from 1 per cent. to 20 per cent., temperatures in the oven from 50° C. to 100° C. and strengths of caustic soda solution from 8 per cent. upwards. In regard to the last factor the maximum swelling is obtained in a 10 per cent. solution. The secret of success lies not so much in the exact chemical

treatment of the fibre sample, but rather in learning empirically the best way of breaking up the fibre under the cover-glass, so that it splits transversely and not longitudinally.

Figs. 1–6, Plates 31 and 32, show sections of fibre prepared by this method and for the most part they are self-explanatory. Sections of the larger fibres are more easy to produce than those of the very small; in the latter there is usually distortion of the individual fibres towards the periphery of the section. Sections of cotton hairs are obtainable only by the dilute acid treatment and even then are seldom perfect; possibly the difficulty is due to the reversal of spiral in the hair structure. The majority of cotton sections appear to be oblique to the hair axis, but occasionally one truly transverse is obtained, and it is of interest to note then that despite the crudeness of treatment the finer details of hair structure are retained. In the original section from which fig. 2 was photographed the daily growth rings could be counted easily. In the sections of hemp, nettle and Asclepias too the concentric structure is well shown.

The lignified strands between the fibres become swollen and blackened although the treatment does not appreciably change the appearance of the cellulose wall. For this reason the sections throw interesting light on the amount and distribution of the inter-cellular material. In section the lignified strands appear as beads between the fibres; they may be seen faintly in the flax section and much more clearly in the Bologna hemp and jute, as would be expected. Ramie, nettle and Asclepias, on the other hand, have a deficiency of inter-cellular material, so much so in ramie that the fibre sections will not cohere in bundle form. In the typically "hard" fibres, manila and banana, all the fibres appear welded into a solid mass.

These sections have the advantage, which will be appreciated by every fibre microtomist, that they are exactly transverse, usually perfectly flat and entirely free from artifacts caused by a razor. Moreover the method is rapid, the section of Bologna hemp, for instance, shown in fig. 3, was prepared from the raw fibre and photographed within the space of 7 minutes.

The process is simple but the actual cause of the formation of sections is perhaps somewhat complex. It may be suggested that the crystallites composing the wall are less easily hydrolysed than the inter-fibrillar part of the fibre. The result of acid hydrolysis is therefore the formation of planes of weakness parallel to the long and short axes of the crystallites. In immersing in caustic soda solution the hydrocellulose, at any rate partly, goes into solution allowing the fibre to be broken up mechanically parallel to the crystal axes. It is clear that besides a concentric spiro-fibrillar structure the fibre also has a

tranversely lamellated structure. It is conceivable that this structure also is related to periodic growth increments as is suggested for the concentric structure. Although nothing definite can be asserted, it is thought that the transverse planes of weakness formed on tendering cellulose fibres bear no relationship to the so-called dislocation marks ; these latter generally appear to be at right angles to the fibrillar spiral and therefore not transverse to the main fibre axis. The transverse planes of weakness are far more numerous than the dislocation marks.

It is interesting to note that segmentation having once started proceeds right through the fibre bundle in the same plane ; there is no evidence of stepping as the fissure passes from one fibre to another, although indeed such change of level, if small, would be difficult to observe in surface view. It seems evident that the lignified inter-cellular thickenings are without lamellated structure ; when segmentation of the fibre takes place the lignified strands snap across only approximately in the same plane as the fibre proper. In a longitudinal view of a fibre from which a section has segmented the broken ends of the lignified strands may often be seen projecting like pegs.

General Discussion.

It is hoped that the method of making fibre sections by chemical tendering may be found of practical use in the routine identification of fibres. The fibre bundle section is always a valuable diagnostic character, but the ordinary methods of preparing sections involve a somewhat troublesome technique and the provision of expensive apparatus. The whole matter of fibre identification is based on rather unsatisfactory tests. Too often reliance has to be placed on staining reactions which are far from infallible. The introduction of any new method, specially if based on a visible and reasonably constant characteristic, is of value.

In this connection it may be suggested that further use might be made of the general appearance of various fibres when tendered, stained with congo red and swollen in caustic soda, or by the viscose reaction ; the differences between fibres are then often very distinctive.

Another characteristic deserving considerably more attention than it has hitherto received is the direction of the drying twist of single fibres. Nodder (5) showed how valuable a characteristic this was for distinguishing flax from hemp at all stages of manufacture. Hitherto it has been supposed that the Linaceæ, Asclepiadaceæ, Apocynaceæ and Urticaceæ were the only families showing a clockwise drying twist of the ultimate fibre. Recently we

have found that *Neoglaziovia variegata* also has a clockwise drying twist in contradistinction to the closely related Bromeliaceous fibre, pine-apple, which has an anti-clockwise twist, an interesting and simple method of distinguishing between the two. It is possible that a careful examination of other lesser known fibres would bring to light more distinctions of this kind.

In conclusion, we would suggest that in place of the rather haphazard methods given in most text-books on the subject, a sound and intelligible key to the identification of all vegetable fibres could probably be based on six simple characteristics, all of which could be determined for a fibre in a short space of time without any complicated technique. These characteristics are: (1) The average length of the fibre strands; (2) the average length and diameter of the ultimate fibre; (3) the direction of the drying twist of the ultimate fibre; (4) the appearance of the chemically formed cross-section of the fibre bundle; (5) the appearance of acid tendered fibres mounted in alkali; (6) a chemical test for lignification.

Our thanks are due to the Linen Industry Research Association for permission to publish this work.

Summary.

A simple method is described for obtaining thin transverse sections of plant fibre bundles by chemical means. The fibre is boiled in sulphuric acid and without washing is then dried in an oven until it commences to char. The tendered fibre is mounted in caustic soda solution and submitted to suitable pressure whereupon the fibre bundles segment into transverse sections. These sections are usually between 10 μ and 20 μ thick, quite flat, exactly transverse and retain all the fine details of structure present in the untreated fibre. It is suggested that sections formed in this way will be a valuable aid to the routine identification of different fibres. Tentative suggestions are made as to the underlying causes of fibre segmentation. Sections of six kinds of fibre are illustrated.

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Fig. 1.

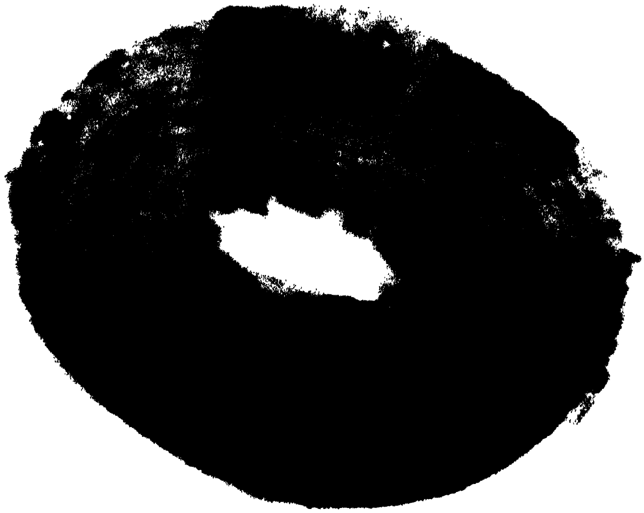

Fig. 2.

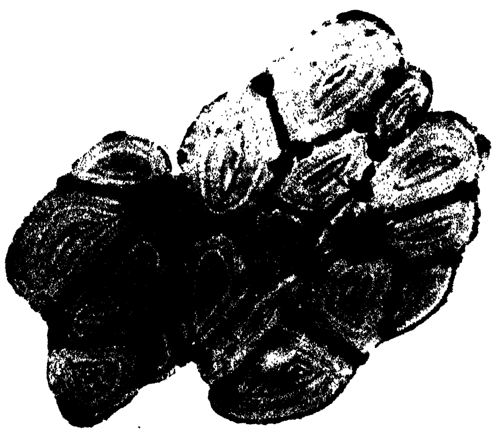

Fig. 3.

(*Facing p.* 362.)

Fig. 4.

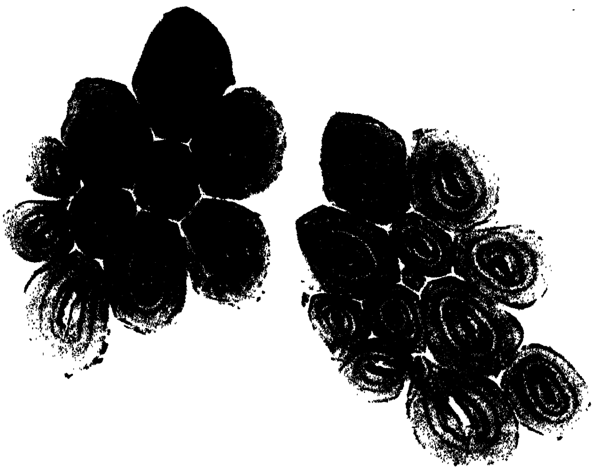

Fig. 5.

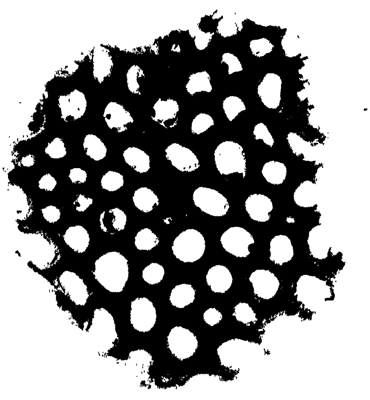

Fig. 6.

DESCRIPTION OF PLATES 31 AND 32.

(All are transverse sections of fibres prepared by the strong acid method described above, with the exception of fig. 2.)

PLATE 31.

FIG. 1.—*Linum usitatissimum*—Flax. × 175.
FIG. 2.—*Gossypium* sp.—Cotton, prepared by weak acid process. × 830.
FIG. 3.—*Cannabis sativa*—Bologna hemp. × 175.

PLATE 32.

FIG. 4.—*Corchorus capsularis*—Common jute. × 175.
FIG. 5.—*Asclepias fruticosa*—African milk weed. × 175.
FIG. 6.—*Musa textilis*—Manila hemp. × 175.

594 . I Pecten : 612 . 816

The Adductor Mechanism of Pecten.

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Introduction.

The adductor muscles of most lamellibranchs fulfil two functions, first the closing and keeping closed of the valves in response to unfavourable or noxious stimuli, and second occasional partial closure while the animal is respiring and feeding and not strongly stimulated. This last is supposed to be a means of cleaning out the mantle cavity. In *Pecten* and a few allied forms the adductor mechanism has been modified for swimming by means of rapid rhythmic movements of the valves. The mechanics of the swimming process have been described by Buddenbrock (1). The movements are brought about by the large single adductor muscle (posterior adductor) situated nearly centrally between the valves. Like the adductors of most lamellibranchs the muscle is composed of two easily distinguished parts. The larger part is yellowish and translucent in appearance, of very soft consistency and composed of striated fibres. It is responsible for the rapid flapping movements of swimming. The other part, which generally constitutes less than 10 per cent. of the total weight of muscular tissue, is white and opaque in appearance, much tougher in consistency, and composed of unstriated fibres. It is the part which is

responsible for keeping the valves closed, and has been called the "catch" muscle (sperrmuskel, von Uexküll) (2). The two portions of the adductor muscle will be referred to as the large or *quick* and the small or *slow* respectively.

If one attachment of the slow muscle is severed without other injury to the animal and the quick muscle is stimulated reflexly, by touching the mantle, rapid flapping movements are obtained just as in the intact animal, but the valves cannot be kept in the closed position against the tension of the elastic ligament of the hinge for more than a few seconds. As soon as excitation ceases the valves gape. If, on the other hand, the quick muscle is severed, leaving the slow, the only reaction obtained with the whole animal is a slow closure of the valves which may continue for some time. These phenomena have long been known (Bronn (3), Coutance (4)). Biedermann (5) in 1885, described what is evidently the twitch of the slow adductor muscle of *Anodonta* but did not consider it a twitch, apparently because it was several hundred times slower than that of frog's skeletal muscle (Biedermann (6), vol. 1, pp. 178, 187). Pavlov (7), however, recognised the nature of the response of *Anodonta* muscle. Prior to the recent work on the mechanical properties of muscle (Gasser and Hill (9), Hill (8), Levin and Wyman (10)), it was evidently difficult for observers to realise that the same fundamental change might underlie such an enormous difference in time scale, a difference due in part at least to differences in the viscous-elastic constants of the tissue. From the experiments to be described it is concluded that the most important difference between the slow muscle of *Pecten* and vertebrate skeletal muscle is a matter of " viscosity."

It must be remembered, however, that *Pecten* in some ways is not a typical lamellibranch. Instead of having one reflex response to noxious stimuli, *i.e.*, prolonged closure, it has also the alternative swimming away, and this involves inhibition of the slow muscle. The development of the swimming reflex and the consequent modification of the nervous system have subordinated the functions of the slow muscle. In fact some individuals refuse to close their valves for any length of time, whatever the reflex stimulation applied. Another consequence of the development of swimming is that the length and range of movement of the adductor is much greater. Swimming needs a greater gape than merely protruding syphons or foot, or admitting water. For these reasons and because of the marked differences between different species of *Pecten*, it may not be safe to argue from *Pecten* to other lamellibranchs.

Of the four different species examined *P. opercularis* (*Æquipecten opercularis*) is the most active swimmer. It has thin light valves about 5 cm. in diameter.

P. maximus has much heavier and larger valves, which may exceed 10 cm. in diameter, and is more sedentary in its habits. *P. varius* is still less active, living generally attached by a byssus. It is rather smaller than *P. opercularis*. *P. magellanicus*, the giant scallop of the Atlantic coast of America, appears to be generally similar in habits to *P. maximus*, but reaches 15–20 cm. diameter.

Anatomical evidence (Dakin (11), (12), Drew (13)) shows that the main motor nerve supply to both parts of the adductor comes from the visceral ganglion lying on the ventral surface of the large muscle and easily seen on account of its yellow colour. Two broad bands of nerves pass backwards across the ventral surface of the large muscle supplying the small part, but these nerves are also a main afferent and efferent tract for the mantle, which is the chief sensory area. In the mantle is found the circumpallial nerve, which contains numerous ganglion cells (Dakin (11), p. 83). No nerve cells have been described in or about the substance of the slow muscle (*cf.* Pavlov (7), p. 25, for similar condition in *Anodonta*) so that if the nerves from the visceral ganglion and the mantle be cut the muscle may be considered to be isolated from the nervous system.

The slow muscle when isolated in the relaxed state survives for many hours in sea water or moist air provided the temperature is low. At temperatures over 15° C. it deteriorates rapidly. The quick muscle, which is too thick to obtain oxygen by diffusion, naturally does not survive so well, but remains excitable sufficiently long for simple observations to be made on it.

Experimental.

Slow Muscle.

Isolation.—The slow muscle of *P. magellanicus*, after the nerves connecting it with the visceral ganglion have been cut, is generally obtained in a fully relaxed condition; that is to say, after a twitch, or after the valves have been held together for a minute or two, it relaxes under the tension of the hinge ligament in 10 or 20 seconds to a length corresponding to the normal gape of the animal while "flapping." By "flapping" is meant the swimming movements, which involves excitation of the quick muscle only and reflex inhibition of the slow muscle. The slow muscle after isolation from the visceral ganglion can no longer be stimulated reflexly by stimulation of mantle or gills and appears to be completely isolated from the animal's nervous system. The cases where the muscle does not relax immediately, as after strong reflex stimulation, are discussed later under the heading of "contracture" (p. 369).

The behaviour of *P. maximus* and *P. opercularis* slow muscle is different.

If *P. opercularis* be induced to flap and the nerves rapidly cut across while the animal is gaping, the muscle can frequently be obtained in a completely relaxed state for a time, but it can still be stimulated reflexly from the mantle. In most cases the response takes the form of a prolonged contracture which may last for hours. Removal of the mantle produces some degree of contracture. *P. maximus* is similar, but the muscle is almost always in a state of contracture after cutting the nerves. Probably the ganglion cells of the circumpallial nerve have a direct connection to the slow muscle in these two species, while in *P. magellanicus* the motor nerves all go *via* the visceral ganglion. Because of this the isolated muscle is most favourably studied in *P. magellanicus*, but apart from this difference in innervation there appear to be only minor differences between the three species.

The slow muscle isolated in the relaxed state shows no signs of spontaneous activity and does not appear to be stimulated by stretching. It can be stimulated electrically to give a slow twitch and when injured goes into a permanently contracted state. It is highly viscous, and at short lengths and under small loads very extensible, so that it takes more than an hour to attain its equilibrium length under these conditions. Consequently the length of the relaxed muscle under no load can only be determined very roughly. From observations of *P. magellanicus* it is concluded that the length *in situ* with valves closed is about the resting length under no tension, so that any opening of the valves brings it under tension. The tension due to the hinge ligament stretches it to the gaping position of the normal animal in about 10 seconds. If left for an hour or more it stretches much further. The gaping position differs considerably in different individuals and species: in *P. magellanicus* it corresponds from 20 to 50 per cent. elongation, in *P. maximus* about 30 per cent., and in *P. opercularis* 50 to 80 per cent. elongation. At the gaping position *P. magellanicus* the tension on the muscle due to the ligament is from 100 to 400 gm., per square centimetre cross-section, calculated on the dimensions at this length. With the valves shut the tension is 300 to 500 gm. per square centimetre, calculated for the shorter length. This tension is about one-tenth of the tension the muscle can develop under reflex stimulation.

For the purpose of these calculations the cross-sectional area is found from the length and weight of the muscle, assuming a density of unity, and it is assumed that all the fibres are parallel to the direction of tension (which is not quite correct). The measurements have been made after removal of the quick muscle, but the differences in tension introduced by the presence or absence of the relaxed quick muscle are probably quite negligible.

The slow muscle of *P. maximus* seems very similar to that of *P. magellanicus*. That of *P. opercularis* is more extensible and all the tensions seem to be smaller, including the maximum tension developed by the excited muscle. Its geometrical form is more complex and there is more obliquity of the fibres. Young animals of the larger species have more extensible slow muscles than older ones have.

Electrical Stimulation.—The slow muscle is relatively insensitive to single induction shocks, but can be stimulated by a rapid succession of strong induction shocks sent in for a few seconds. Continuous faradic stimulation does not result in a contraction appreciably longer than a single twitch, but by interrupted faradic stimulation a tetanic contraction lasting a few minutes can be obtained. Even under these conditions excitability falls off, but it revives again after a few minutes interval. If a sufficient interval is allowed to elapse a large number of twitches can be obtained before fatigue appears. The rapid loss of excitability during the passage of induction shocks does not appear to be due to injury or fatigue. The tensions obtained by electrical stimulation have not been as large as those developed under reflex stimulation, so that they cannot be maximal. For instance in *P. magellanicus* 4 kg. per square centimetre was the highest tension observed to be developed reflexly, against 3·5 kg. per square centimetre by direct stimulation. In *P. maximus* the discrepancy is greater; the highest reflex tension was 6 kg. per square centimetre against 3 kg. per square centimetre by direct stimulation. As, however, the muscle stimulated directly was not completely relaxed and was less excitable the conditions were less favourable.

Make-and-break of constant current excites the slow muscle, which shows a definite and reproducible threshold. However, to obtain excitation of any large number of fibres by constant current, considerable quantities of electricity have to be passed through the tissue, and polarisation can hardly be avoided. The methods of stimulation used hitherto are not entirely satisfactory.

In an isometric twitch at ordinary temperature, the latent period is about 0·2 second, contraction occupies 2 to 5 seconds and relaxation 10 seconds or more. The relaxation time is determined by the viscosity of the muscle, as the drop in tension after a sudden stretch follows the same curve. Similarly the rise in tension after a sudden release during the contraction phase follows the same curve as the initial rise in tension. On a time scale about 100 times slower the phenomena resemble those observed by Gasser and Hill (8), in frog's muscle, except that there is no marked change in viscosity of *Pecten* muscle produced by excitation. The absence of change in viscosity is seen

not only in twitches produced by direct stimulation which probably do not involve many fibres, but also under reflex excitation when higher tensions are developed (fig. 1). In its time relations the muscle is evidently similar to

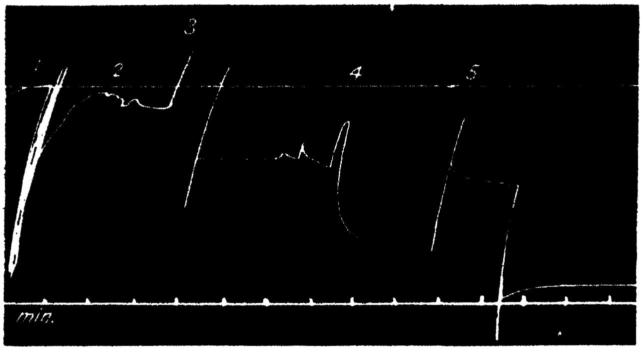

FIG. 1.—*P. magellanicus*: Isometric. *Top line*—tension about 4 kg. per sq. cm. on slow muscle. *Bottom line*—tension of ligament only, about 0·4 kg. per sq. cm. Minute intervals marked in. Intact animal connected to tension lever. (1) Mantle stimulated, 3 flaps, then contraction of slow muscle; (2) Nerves cut; (3) Quick stretch. Between (3) and (4) release to original length, electrodes put in place; (4) Short faradic stimulation; (5) Quick stretch, similar curve to (3). After release to original length muscle is relaxed, *i.e.* tension at the end the same as after a flap. There is considerable "kick" of the writing point after sudden movement.

Holothurian body muscle (Hill (9)), but is much less extensible and is capable of developing much higher tensions.

The records of movement obtained by sudden loading and unloading are similar to those of Winton (14) with *retractor penis* muscle, in that they are not exponential, as the Levin-Wyman (10) theory predicts, but show a flat portion of uniform rate of elongation, which may be due as Winton suggests to a pure viscous element (fig. 2).

A very striking demonstration of the viscous-elastic properties of the muscle is obtained with a relaxed isolated muscle, left *in situ* between the valves, which has been stretched by the hinge ligament-tension. On pushing the valves together the muscle at first hangs limp but in half a minute or so pulls itself together. On releasing the valves after a few minutes they open a little and then remain so nearly stationary that the movement is inappreciable to the eye. In the end, of course, they gape to the original equilibrium position. It is undoubtedly such phenomena observable with the isolated muscle or the intact animal that led observers in the past to attribute a mysterious "tonus" mechanism to the muscle. Prior to the experiments of Gasser and Hill (8),

FIG. 2.—*P. magellanicus*: slow muscle, movements against tension of hinge ligament. *Bottom line*—muscle 44 mm. long. *Top line*—31 mm., valves shut 28 mm. long. Time scale as fig. 1. (1) Loaded = about 440 gm. per sq. cm. on muscle; (2) Unloaded; (3) Short faradic stimulation.

it would have been hard to realise that any viscous-elastic body would do the same thing, though if, like frog's muscle, it is much less viscous the movements are too rapid to be directly visible.

Chemical Stimulation.—Unlike mammalian smooth muscle the slow muscle of *P. opercularis* is unaffected by large concentrations of adrenalin (1 : 100), histamine (1 : 1700) or acetyl choline, and the behaviour is not readily altered by asphyxia or by sodium cyanide (M/500). This muscle is also extremely insensitive to the cation content of the saline solution. Variations in calcium and magnesium appear to have no action, but an increase in potassium concentration has a stimulating action, as it has on nearly every type of muscle. With varying potassium concentration the rate of increase of tension is more affected than the final tension reached. The potassium effect is reversible.

"*Contracture*" *after Reflex Stimulation.*—If the muscle of *P. magellanicus* be isolated after strong reflex stimulation it generally relaxes extremely slowly, taking an hour or more to relax to an extent that takes a few seconds after a twitch, and it may maintain a steady tension for many minutes. The condition of prolonged contraction is here called "contracture," without thereby implying any theory as to its nature or origin, or that it is necessarily a process falling within Gasser's (15) definition. The "contracture" can be much diminished or abolished by brief faradic stimulation, which produces a small contraction followed by relaxation along the normal curve (fig. 1). This phenomenon was brought to our notice by Dr. E. Bozler, who observed it in *P. opercularis* in 1928, but what seems to be the same phenomenon was described

by Pavlov (7) in *Anodonta* and attributed by him to the presence of inhibitory nerves. It is, however, more conspicuous and more regularly obtained in *P. magellanicus* than in *P. opercularis* or *P. maximus*. In the two latter species some " contracture " generally remains and the extent of relaxation after the first direct stimulus on the muscle may be very small. Subsequent stimuli have no effect. The " contracture " is not accompanied by increased viscosity, because the curve of fall of tension after sudden stretch is similar to that of relaxed muscle. The tension developed in " contracture " in *P. maximus* after strong reflex stimulation may be more than sufficient to close the valves against the hinge ligament, but is probably never as much as one-fifth of the maximum tension obtainable during reflex stimulation. The greater the " contracture " the less excitable the muscle. The contracture diminishes in an hour or so, but in *P. maximus*, where it is very marked and long maintained, the muscle is generally dead before it has passed off. " Contracture " in *P. magellanicus* may start with a high tension, but this soon falls off to quite small values (fig. 1).

It might be suggested that there are special fibres in the muscle giving an extremely slow response and unaffected by the electrical stimuli which excite only the quicker fibres ; but the fact that a relaxation of rapidity similar to that of a normal twitch can be obtained by stimulating a muscle in " contracture " (as described above) disposes of this suggestion. It is possible that the " contracture " is produced by excitation through special nerve endings. If so, the nervous path in *P. opercularis* and *P. maximus* would appear to be the direct path from the mantle ; but since contracture is obtained in *P. magellanicus*, where this path is absent, it seems more likely that contracture is the result of a special kind of excitation rather than a special type nerve ending.

Assuming that the stimulus producing " contracture " comes through the same nerves to the same muscle fibres producing the ordinary type of contraction, there are still two possibilities open. The effect may be due to a prolonged state of excitation, something like the veratrine effect in frog's muscle, or the state of excitation may have ceased, but the architecture of the muscle fibre may somehow have got " stuck " at a length and tension corresponding to the contracted state, instead of passing over at once to the relaxed state. In this case the molecular configuration corresponding to the excited state of the fibre would be maintained without a continual fresh supply of energy. The state of " contracture " would then be a state of " tonus," in the special sense in which the word has sometimes been used, and the muscle

would possess a "catch" mechanism. On this view the effect of a stimulus in abolishing the state is not an inhibition, as there is nothing to inhibit, but it would be due to a stirring up, as it were, of the architecture of the muscle fibre, so that it could return to the ordinary relaxed state.

If, on the other hand, the contracture is the result of a prolonged state of excitation, whether intrinsic in the muscle fibres themselves or due to nerve endings, there will be a continued output of energy and the state of affairs will not differ fundamentally from that of tetanic contraction. The effect of a stimulus in abolishing contracture may therefore be due to special inhibitory nerves, as Pavlov (7) supposed; his supposition, however, does not seem to be a necessary conclusion. Relaxation always follows a contraction, though it may be a small one, and seems to be the consequence of the fresh excitation rather than inhibition pure and simple. As faradic stimulation produces diminished excitability, that in itself might be enough to account for the relaxation.

If a "humoral" theory of excitation and inhibition be accepted, a state of excitation that lasts for a long time after cutting the nerves offers no great difficulties. It implies simply that the exciting substance must be under these circumstances unusually stable or present in unusually large quantity. If, on the other hand, the exciting agent to the muscle fibre be the action current of the nerve operating at the nerve endings, the alternative view that there is no continuous state of excitation would be more acceptable. The question could be settled by measurement of the heat production, but that is hardly possible so long as the state of contracture can be produced only by reflex stimulation.

Quick Muscle.

The quick muscle is always completely relaxed except immediately following excitation. A single shock from an induction coil produces a rapid twitch, which does not differ appreciably from a reflex contraction in the intact animal. In *P. opercularis* the movements of contraction and relaxation against the hinge ligament tension each occupy about 1/10 second at ordinary temperatures. In the large animals with more massive moving parts the movements are not so rapid. On account of the fragile nature of the tissue and the very large tensions involved, tension records have not been obtained.

Repeated stimulation very soon renders the muscle inexcitable, but there is some recovery of excitability after a few minutes pause. The total number of twitches that can be obtained by direct electrical stimulation is of the same order as the number obtained by strong reflex stimulation. The total number

of twitches obtainable in different species, and the amount of lactic acid found, vary together as shown in Table I.

Table I.

Species.	Average Stimulation maximum Lactic Acid.	Number of twitches.
	Per cent.	
P. opercularis	0·10	50
P. maximus	0·05	30
P. varius	0·04	20

This difference in the amount of work the muscle can perform may be an intrinsic property of the species; on the other hand it may be the effect of different training in animals with different habits. *P. opercularis* swims far more in the course of its life and may be supposed to develop its muscle more. This view is supported by the fact that young specimens of *P. maximus* have usually more active muscle than older animals with heavier valves.

Reflex Movements.

The adductor mechanism is capable of three types of response, according as quick muscle only, slow muscle only, or both muscles are excited. Of the first type are the swimming movements, of the second are the movements of the animal when quiet and " feeding " with valves partly open and also the condition in prolonged closure. The third type is obtained occasionally as a response to noxious stimuli. It consists of a rapid twitch of the quick muscle and simultaneous excitation of the slow muscle, which comes into action in time to prevent any appreciable opening of the valves when the quick muscle begins to relax. The valves are then held by the slow muscle alone for a longer or shorter time (fig. 4). This is probably the primitive lamellibranch response to noxious stimuli and is the only one most forms are capable of giving. *Pecten* has the alternative of swimming away.

The normal swimming movement is preceded by a wide gape which lasts for a few seconds. The gape seems to provide part of the reflex stimulus, because if the opening movement is stopped half-way the closure usually fails to follow, but does follow when the valves are again allowed to open fully. The reflex is mediated by the visceral ganglion and is abolished by injury to that organ. It is more readily abolished by anæsthetics than the slow-muscle reflexes.

Slow-Muscle Reflexes.—Records have been made of the normal movements of

P. magellanicus in the laboratory tanks (figs. 3 and 4). The movements consist of a partial closure at the speed characteristic of the slow muscle twitch,

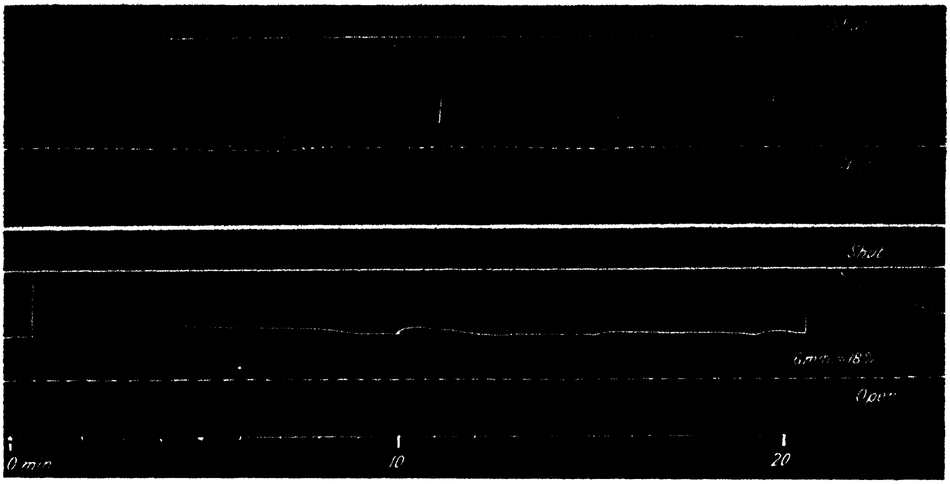

FIG. 3.—*P. magellanicus* : spontaneous movements. Large sluggish individual undisturbed. Lever magnification × 3·6. *Top line*, valves shut. *Dotted line*, 18 per cent. elongation of muscle, in this animal about equal to elongation of relaxed isolated muscle under tension of ligament.

followed by a gradual and irregular relaxation, then another partial closure, and so on. The interval between closures may be half-an-hour but is more usually 5 to 15 minutes. The amplitude varies from a hardly perceptible movement to nearly the whole range of normal movement and the muscle may be fully relaxed between closures.

Different animals tend to reproduce characteristic curves. In some cases a closure was seen to follow a definite stimulus, such as vibration of the tank or turning off the light. Animals repeatedly excited gave more frequent movement (5 closures in 10 minutes). In some cases the muscle was maintained at constant length for several minutes, but more generally it was moving throughout. The whole process suggests stimulation by a succession of volleys of impulses of gradually diminishing frequency.

When *P. magellanicus* is removed from the water it is seldom possible to obtain prolonged closure of the valves; the animal is liable to flap and then gape, particularly if the tension on the muscle is increased by pulling the valves apart. *P. maximus*, however, will maintain high tensions for a long time, and tension records have been obtained from it. A steady tension is not usually

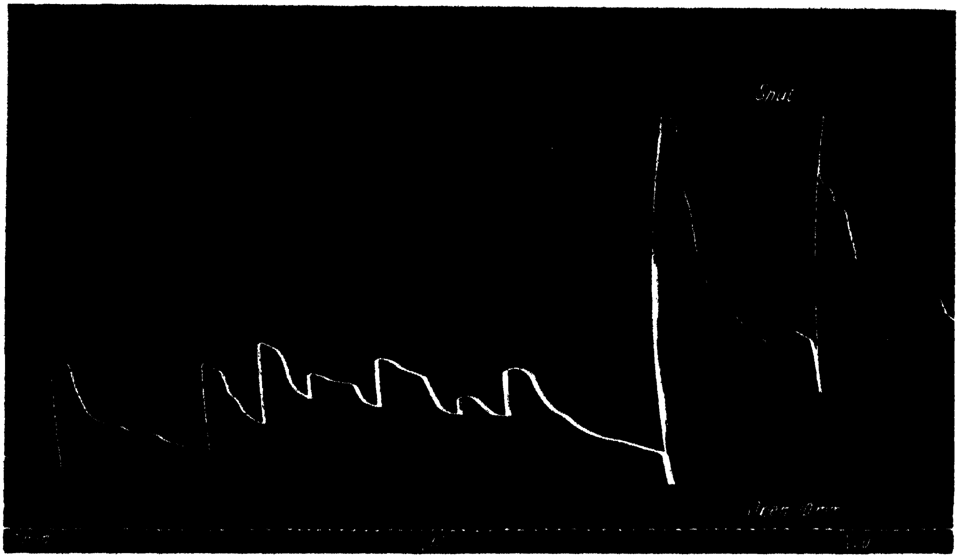

FIG. 4.—*P. magellanicus*: spontaneous movements. Small lively animal. First part of record, excited by small fish swimming near. Last part, effect of switching off light (×). Two flaps are shown.

Lever magnification × 8·0. Top line, valves shut. Dotted line, 10 mm. elongation; probably about 30 per cent.; muscle length not measured.

maintained, but more frequently a slow irregular fall with occasional sudden increases, suggesting a condition similar to that found in the quiet animal but operating at higher tensions in the more excited condition. The phenomena suggest simple tetanic contraction of the muscle, but do not, of course, exclude the possibility of "contracture" being present as well.

Cutting the nerves from the ganglion produces relaxation of the muscle, varying from the complete relaxation often obtained in *P. magellanicus* to partial relaxation with residual "contracture" usual in other forms. In the intact animal (*P. maximus*) stimulation of the left-hand band of nerves produces contraction of the slow muscle; stimulation of the right-hand nerves frequently gives partial relaxation. The effect is evidently due to stimulation of afferent nerves, because, after cutting the nerves between the electrodes and the ganglion, the effect (if any) is the same on both sides and is merely a small contraction. The experiment therefore throws no light on the mechanism of inhibition.

Von Uexküll (16) found that stimulating the right cerebro-visceral commissure produced relaxation, of the left contraction. This result, too, which has been confirmed, depends upon nerves afferent to the visceral ganglion

The two observations merely show a difference in function of the two sides of the ganglion.

Summary.

(1) The slow part of the adductor muscle can be isolated from the nervous system in a completely relaxed state in *P. magellanicus*. Stimulated electrically it gives twitches about 100 times as slow as frog's skeletal muscle, which can be fused to form tetanus. The difference in time scale is determined by the viscosity of the tissue, which in this muscle is about the same in the excited and unexcited states. The tensions developed are large.

(2) In *P. maximus* and *P. opercularis* different nervous connections make it difficult to isolate the muscle without some " contracture " ; apart from this the muscles are similar to that of *P. magellanicus*.

(3) The state of " contracture " is a result of reflex excitation which survives isolation, but may be partly or completely abolished by direct faradic stimulation. The " contracture " is not accompanied by increased viscosity. It is uncertain whether it is due to continued excitation or not. The tensions in " contracture " are much less than the maximum tensions obtainable.

(4) Reflex movements of intact animals suggest that for the most part the muscle is contracting tetanically.

(5) The quick muscle gives a rapid twitch with single induction shocks that resembles the normal reflex contraction. Complete fusion of twitches is not readily obtained. The maximum number of contractions obtained by stimulation is not large and varies in different species according to the animal's normal activity.

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On the Histological Evidences of the Organic Content and Reactions of Marsupial Enamel, with a Note on Human Enamel.

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[Plates 33-36.]

CONTENTS.

Part I.—Introductory and Historical.

During histological investigation of the organic content of human enamel it was noticed that portions of young human enamel (Plate 33, fig. 1) which had been permeated by stain from the dentine aspect were permeable through about three-fourths of their thickness and so looked structurally very similar to marsupial enamel treated in the same manner (fig. 2), the chief differences being the direction of the prisms, and the degree and regularity of patency of the inter-prismatic substance. As histological structure is more readily appreciated in marsupial than most other mammalian enamels, owing to its tubularity, it was decided to make a brief examination of the enamel of young and old marsupials of the genus Macropus, for comparison with similarly made preparations of human enamel. Macropods were chosen because they exhibit the greatest degree of tubularity among marsupials.

Sir John Tomes (1) in 1849 first described that tubular enamel occurs in marsupials and was " common to the teeth of at least the great majority of

marsupials, if not all, except the wombat." He also showed (2) that when it was decalcified the tubes could be seen maintaining their continuity with the dentine after the lime salts had been removed. He, too, first noted (1) that "in the human teeth the dentinal tubes are in small numbers, occasionally only continued for a short distance into the enamel," and so drew attention to a resemblance between human and marsupial enamels at the amelo-dentinal junction. The observations of J. H. Mummery (3) in 1914 confirmed this.

Sir Charles Tomes (4) believed that these tubes were due to persistence of the process (described by and named after Sir John Tomes) of the ameloblast cell, which he thought remained uncalcified in the central portion of the finished enamel prism in marsupials, but became calcified almost at once in human and most other mammalian enamels.

Von Ebner (5) in 1890 described and figured that the tubes lie between the prisms; this has since been confirmed by others and is now the generally accepted view.

Part II.—Nature of Material used and Technique of Investigation.

As it was desired to examine the enamel under conditions as near those obtaining during life as possible only recent material was used. This was obtained from a young female Aru Island wallaby (*Macropus brunii*) and an old male black-tailed wallaby (*Macropus ualabatus*); newly erupted teeth were obtained from a young museum specimen of the latter species.

The method of investigation was to permeate the enamel by stains, which were used in two ways :—

I. For permeation of the enamel from the pulp canal, so that stain had to traverse the dentine before it had access to the deep aspect of the enamel.

II. For lateral permeation.

In addition a few specimens were permeated with ammoniacal 5 per cent. silver nitrate, and the silver then precipitated *in situ* by adding a solution of slightly alkaline sodium hydrosulphite. This, however, did not cause precipitation to occur throughout tissues, and it was ultimately found better to grind the section from the permeated tooth, using xylol, as described later, and then precipitate the silver by exposing to sunlight. In some teeth the protein content was digested with 1 per cent. trypsin before permeating with the silver salt, as it was thought that the protein might be coagulated by the salt and so interfere with permeation, but in practice no appreciable difference was

noticed in sections prepared in this manner. Further experience with this technique is desirable. It was hoped that the smaller molecule of the crystalloid salt would penetrate better than the stains and show greater detail, as indeed it does.

For method I, the root portion of each tooth was cut off transversely and the pulp removed. Solid stains soluble in aqueous media were then introduced into the pulp canal and moistened with normal salt solution; the opening was then closed with oxyphosphate of zinc cement and the tooth placed in normal salt solution. Only the procumbent lower incisor teeth were suitable for this method.

For method II, the teeth were cut into thick sections and placed in a strong solution of stain in normal salt for several days before being ground thin. This was done to see, by comparison with method I, if additional stain would penetrate the enamel from its now exposed lateral aspects. Cheek teeth were used for this purpose.

Methylene blue and acid fuchsine were used for permeation; both permeated well, but it was found difficult to take photomicrographs in colour which showed accurately the area of permeation by methylene blue; moreover, this stain is not always fast in Canada balsam.

It was hoped that these stains would find their way through uncalcified spaces in enamel by the physical process of diffusion; should such diffusion occur under method I, one would infer that they had permeated a space occupied in life by some vital fluid, since it is believed there are no vacuous spaces in body tissues. It is known that such vital fluids occupy uncalcified portions of dentine, and stains would have to traverse these before they could reach the enamel; moreover, these "spaces" in dentine can be seen in direct continuity with the enamel. Should diffusion appear in continuity from the pulpal aspect of the dentine to the external aspect of the enamel, one would infer that this was due to traumatic injury, since it is inconceivable that there should be physiological leakage of vital fluids into the mouth.

It was found that all the stain that was going to diffuse into the enamel did so within a few hours of introduction. The teeth were not allowed to become dry, as artefact cracks frequently occur in all calcified tissues of dry teeth; this is seen in many museum specimens (fig. 3). For this reason specimens were kept in aqueous saline during the permeation process, otherwise permeation might have been due to capillary attraction and not diffusion. Alcoholic stains were not used, in case such hygroscopic medium might produce artefacts by abstraction of water.

A difficulty presented itself in grinding sections. When grinding hard tissues it is usual to keep the carborundum wheel used for this purpose wet, to avoid the heat of friction and consequent damage to tissues; the stains used, and the silver salt, being freely soluble in water, would, of course, dissolve out when the wheel was wetted, and this occurred even when reagents reputed to fix the stains were used.

The difficulty was overcome by using for this purpose a fluid in which the stains were insoluble. Xylol was tried, and met requirements very satisfactorily; no stain is lost by this method and a stain-diffused tooth may be taken direct from a watery medium, dried on a cloth and ground. When ground sufficiently thin it is washed in xylol and mounted in Canada balsam. The absence of water made the tissues rather more brittle than when making a section in the usual manner. The teeth used were not removed by forceps, so one may conclude that no artefact cracks were produced before permeation.

Part III.—Histological Appearances and Inferences drawn therefrom.

Young Marsupial Enamel (*Macropus brunii*).—The cheek teeth of this animal showed only slight signs of wear. In teeth prepared by method I the enamel was found to be freely permeable *via* the dentine (fig. 4) to three-fourths or more of its extent (fig. 2). This permeability appeared to be *via* the inter-prismatic substance, though occasionally prism substance seemed to have absorbed some stain. That permeability was inter-prismatic was confirmed by making a section transverse to the long axis of the prisms (fig. 5). This section was made from a cheek tooth by method II.

Old Marsupial Enamel (*Macropus ualabatus*).—The cheek teeth of this animal showed considerable signs of wear, enamel being entirely worn away from large areas of the occlusal surfaces. In sections prepared by method I enamel was hardly permeable, or only so to a slight degree (fig. 6). The tubes appeared to be fewer and narrower (fig. 7), and though not so permeable their continuity with the dentine was still quite evident; one infers that as age and wear progressed, increased calcification of the inter-prismatic substance took place, till it became impermeable and a more or less solid mass.

The above are, however, general statements, because examination of the cheek teeth of the young animal showed (fig. 8) that though the enamel on some aspects was freely tubular through almost its whole extent, on others it was not so, and in these usually showed no trace of its original tubular structure. These latter portions (fig. 9) therefore closely resembled the bulk of human enamel in structure and presented a large number of markings ("laminæ")

which, there can be but little doubt, are mostly cracks, produced by the trauma of mastication. As, however, in human enamel there is evidence that some laminæ may be developmental in origin, the same may be true of marsupial enamel; but when the greater number of laminæ are found on those aspects where the enamel is thin and which are more exposed to trauma, and all stages of reaction, ultimately rendering them impermeable, are observed, it is strong evidence that most are traumatic in origin.

These laminæ or cracks vary considerably in extent and appearance, and inferences of some importance may be drawn from these facts. Some of them extend through the whole thickness of enamel and into the dentine, and of these :—

(i) In some (fig. 10) the dentine is found to have taken in more stain at the point of injury than elsewhere; in these the stain penetrates the extreme extent of the enamel crack, so that there is a potential leakage of lymph into the mouth; one infers that the damage was recent and so gave more ready ingress to stain.

The minute anatomy of the damaged area is seen in better detail in silver preparations and presents remarkable appearances (fig. 11). In these the dentine tubes are larger, more branched and more patent than in the surrounding dentine; it appears as if the initial dentine reaction were analogous to that of inflammation in soft parts, and that this increased patency is for the purpose of bringing an increased amount of calcium salts to the damaged area for its repair, to result ultimately in the production of a scar of increased calcification, known as a translucent zone.

In the enamel the inter-prismatic substance admits more stain in the immediate vicinity of the crack than elsewhere, and in view of subsequent findings (see figs. 13 to 20) it seems certain that this greater patency is a reaction to injury.

If these inferences are true they are of great interest, because they are conclusive evidence that the exchange of calcium salts goes on in enamel and dentine, governed from the pulp. Till now, such exchange has been hypothetical, though it has been recognised for many years that additional calcium salts may be deposited in dentine, and recently in human enamel also, under pathological conditions (6).

(ii) In a second variety (fig. 12, crack 1) a definite reaction is found in the dentine, which has resulted in the production of a translucent zone of

hypercalcification, so that at that point it is impermeable, except centrally, where a few dilated tubes are seen; these are in direct continuity with the enamel crack, which also is permeable. This indicates a reaction of repair, appearing primarily in the dentine (by this method of preparation).

(iii) In a third variety (fig. 13) the enamel crack is permeable mesially for a certain distance and continued to the surface as an impermeable lamina or linear translucency; the dentine shows a translucent zone permeable centrally in continuity with the enamel crack as before.

(iv) In a fourth variety (fig. 14) not only is the enamel crack completely impermeable but the adjacent enamel as well; it shows a definite zone formed by hypercalcification of the inter-prismatic substance, which thus renders it impermeable, and in extreme cases converts it into a non-tubular structure simulating human enamel. A translucent zone is present in the dentine. At this stage there are no centrally dilated tubes present; apparently the purpose for which they became dilated having been achieved, they also have become occluded.

In transverse section (fig. 15) one can show that hypercalcification in the repair of enamel cracks takes place in the inter-prismatic substance, because adjacent to cracks it is seen to be impermeable.

(v) In a fifth variety (fig. 16), possibly when the enamel crack was a small one, repair is of such perfection that a translucent line of hypercalcification is seen, like a linear scar.

After repair has fully taken place in enamel and dentine there is a possibility that translucent zones may disappear, because occasionally impermeable enamel cracks are seen unassociated with translucent zones either in enamel or dentine; in the latter case this may be because the crack never extended into the dentine. Again sometimes, though translucent zones have obviously been present both in enamel and dentine (fig. 17), they are permeable, though not to the same extent as in adjacent uninjured tissues. There is, moreover, the evidence, as seen in such injured areas as in fig. 11, of dentine tubes opened up amidst an impermeable area and of the reopening of inter-prismatic spaces in enamel.

Other cracks, which involve enamel only, are seen, and these, too, may be found permeable in different degrees, as follows:—

(i) Permeable to stain throughout their length, apparently recent cracks (fig. 12, crack 4). Silver preparations (fig. 18) show the opening up of adjacent inter-prismatic spaces, analogous to what is seen in newly damaged dentine and already shown in fig. 11, but the two cracks here seen are at a later stage. The peripheral portions show the commencement of the formation of the translucent zone after the opening up of the inter-prismatic channels, consequent on injury. This proves that repair takes place from without inwards and must therefore be governed from the pulp. In one of the cracks it can be seen that the violence causing it detached a small piece of enamel, and in the other the peripheral portion of the crack has already closed. These preparations also show that there is an accompanying local reaction in the dentine, even though the damage does not extend into it.

(ii) Permeable only through the mesial portion and continued to the periphery as an impermeable lamina or a linear translucency (fig. 12, crack 3, and fig. 19).

(iii) Obvious cracks, entirely impermeable to stain and apparently entirely healed (fig. 12, crack 2).

(iv) Linear translucencies, which are by analogy linear translucent scars (fig. 20).

It will be noticed constantly in the same section (fig. 8, see also figs. 9, 12, 16) that on those aspects of a tooth which present many cracks, the processes of repair and hypercalcification have obliterated the tubular appearance of the enamel. Probably this is aided by the conjunction of translucent zones; whereas on aspects protected from trauma by contact with teeth in front and behind, there are few or no cracks, and the tubular system is typical and extends practically to the surface of the tooth. One inferred that the former aspects were more subject to the physiological trauma of mastication, and hence damage; and in fact this is so. This section is made from a cheek tooth of the young animal, and it is the exposed buccal and lingual aspects which show many cracks and non-tubular enamel. Examination of the enamel covering these aspects of newly erupted cheek teeth of a young *Macropus ualabatus* showed that they are as freely tubular as any other part of marsupial enamel before they have been subjected to the trauma of mastication.

It is a significant fact that if any lamina can be traced through the whole thickness of the enamel, any part of it which is impermeable is always the peripheral portion, unless the damage at that point was rather extensive.

Concurrent with the changes due to age and wear already tabulated there are, in addition, changes in the prism substance. That this becomes hypercalcified is evident, because in the more highly calcified specimens the undamaged portions admit no stain by any of the three methods used.

Part IV.—Conclusions and Note.

From the above data one may therefore conclude that in marsupial enamel: –

1. The tubes are inter-prismatic and consist of spaces of different and varying degrees of patency.
2. In the young animal these spaces are much more permeable than in the old animal.
3. As age and wear progress the enamel becomes more highly calcified. This is brought about partly by deposition of calcium salts occluding the inter-prismatic channels. This, though not at first obliterating the tubular appearance, lessens the number and diameter of these spaces; later, as calcification progresses, they may become obliterated.
4. The enamel is frequently cracked; these cracks often extend into the dentine and occur more on the exposed aspects of teeth than others. Doubtless, these are due to the trauma of mastication.
5. The cracks, though often permeable to stain, appear to become occluded both in enamel and dentine.
6. Since in partly permeable cracks, extending through the thickness of the enamel, it is the peripheral portion which is occluded, closure must be governed from the pulpal aspect.
7. When both enamel and dentine are injured, the dentine reacts locally by an increased patency of its tubes at the site of injury. Ultimately hypercalcification occurs there, but the central tubes leading to the enamel crack maintain their patency till the enamel crack is occluded.
8. The enamel adjacent to its site of injury appears to react in a manner analogous to that of the dentine; it shows increased inter-prismatic patency at first and hypercalcification later. This hypercalcification takes place from without inwards, and commences before the actual occlusion of the enamel crack.
9. Closure of cracks appears therefore to be brought about by calcium salts conveyed *via* the dentine.

10. The above facts indicate that the spaces permeated by stain can in life convey calcium salts for repair and remove them as required.
11. Exchange of calcium salts therefore can take place both in enamel and dentine, this exchange being governed from the pulpal aspect.
12. By analogy with human enamel, some laminæ may be developmental in origin, but, in any case, only such as do not permit of escape of vital fluids into the mouth, since it is inconceivable that there should be such physiological leakage of vital fluids. One can, however, find no evidence of developmental laminæ in marsupial enamel.
13. Marsupial enamel must therefore be regarded as a living tissue which reacts to injury and undergoes increased calcification as age and wear progress.
14. Inflammation is defined as the reaction of living tissue to injury. Apparently both dentine and enamel react to injury, but, being avascular, do not show the usual signs and symptoms of inflammation, as seen in vascular tissues.

Allowing for structural and calcific differences, similar reactions to those shown in marsupial teeth have been found by the author in human enamel and dentine. At present five figures only illustrating these conditions are shown (figs. 21, 22, 23, 24, 25). It follows, therefore, that many of the conclusions concerning marsupial enamel must also be true of human enamel.

My thanks are due to the Zoological Society of London for supplying me with material as it became available, so expeditiously that only a few hours elapsed begween the deaths of the animals and the preparation of the teeth for examination ; also to the British Museum (Natural History) for specimens of newly erupted teeth. My thanks are also due to the Medical Research Council, under whose auspices this work has been done.

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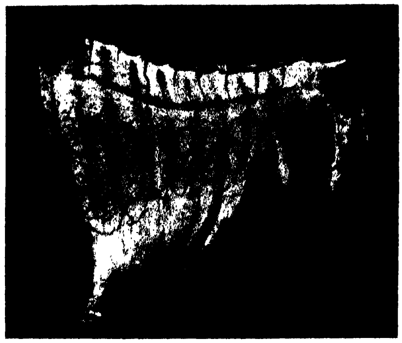

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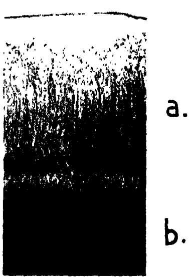

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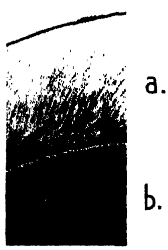

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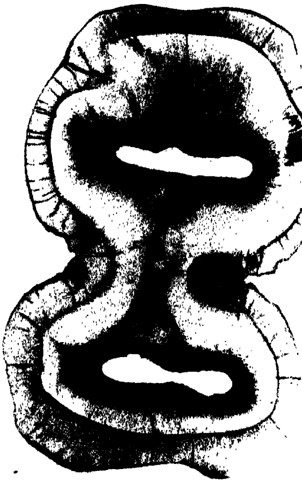
e

d.



c.

f

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a.

b.

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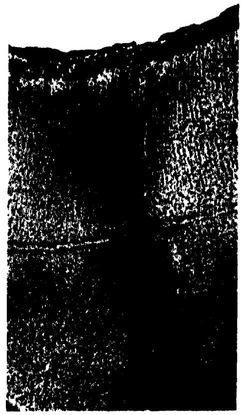

a.

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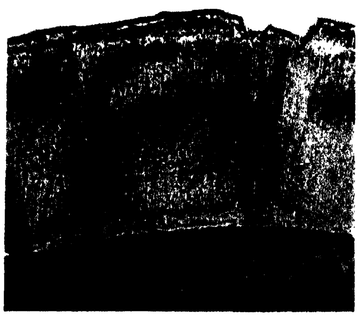
11

a.

b.

15



a.

b.

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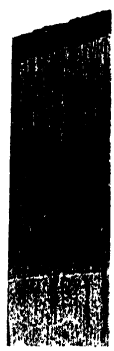

a.

b.

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(5) Von Ebner, V., "Strittige Fragen über den Bau des Zahnschmelzes." 'Sitzungsberichte d. K. Akad. d. Wissensch., Math.-naturw. Cl., Wien.,' vol. 99 (abth. 3), pp. 57–104 (1890).
(6) Mummery, J. H., "Translucent zones in enamel." 'British Dental Journal,' vol. 47. No. 9, pp. 473–482 (1926).

DESCRIPTION OF PLATES 33–6.

PLATE 33.

Lettering applicable to all figures :—*a*, enamel; *b*, dentine; *c*, lingual aspect; *d*, buccal aspect; *e*, mesial aspect; *f*, distal aspect.

FIG. 1.—Young human enamel from a permanent molar, permeated from the pulp chamber by methylene blue; longitudinal section; permeation is seen to be inter-prismatic. × 235.

FIG. 2.—Young marsupial enamel from an incisor, permeated from the pulp chamber by acid fuchsine; longitudinal section, a portion of fig. 4; permeation is seen to be inter-prismatic. × 235.

FIG. 3.—Indian elephant molar, old museum specimen; shows many large artefact cracks. × 2/5.

FIG. 4.—Young marsupial lower incisor, permeated from the pulp chamber by acid fuchsine, longitudinal section; shows degree of permeation in uninjured enamel. × 24.

FIG. 5.—Young marsupial enamel from a cheek tooth, permeated as a thick section from all sides by acid fuchsine, transverse section; the enamel is ground transverse to the long axis of its prisms; permeation is seen to be inter-prismatic. × 235.

FIG. 6.—Old marsupial lower incisor, permeated from the pulp chamber by methylene blue: transverse section; shows degree of permeation in uninjured enamel. × 24.

FIG. 7.—A portion of the enamel of fig. 6. × 235.

PLATE 34.

FIG. 8.—Young marsupial cheek tooth, permeated as a thick section from all aspects by acid fuchsine, transverse section; shows many cracks and non-tubular enamel at *c*, *d*, and few or no cracks, and the tubular system throughout nearly its whole extent, at *e*, *f*. × 11.

FIG. 9.—A portion of fig. 8, *c*, showing obliteration of tubular system, and so looking very like the bulk of human enamel. × 100.

FIG. 10.—See Plate 35.

FIG. 11.—Young marsupial cheek tooth, permeated from all aspects by 5 per cent. silver nitrate. The metallic silver has been precipitated *in situ*; shows a crack extending through the enamel and into dentine permeable throughout its extent. There is increased patency of dentine tubes at the site of injury and opening up of inter-prismatic substance of enamel adjacent to the crack. × 125.

FIGS. 12–14.—See Plate 35.

FIG. 15.—Young marsupial cheek tooth, permeated as a thick section from all aspects by methylene blue; transverse section, with enamel ground transverse to the long axis of its prisms; shows a crack in the enamel; the inter-prismatic substance adjacent is impermeable to stain. × 150.

FIG. 16.—A portion of fig. 8, *e*, shows an occluded enamel crack which remains as an impermeable line of hypercalcification. There appears to have been a slight similar reaction in the immediately underlying dentine; shows also the degree of tubular penetration on protected aspects. × 110.

FIG. 17.—See Plate 35.

FIG. 18.—Young marsupial cheek tooth, from same section as fig. 11; shows two cracks through the enamel. In one (right) the force causing the crack detached a small portion of surface enamel; this crack is permeable throughout its extent. Near the dentine the inter-prismatic substance adjacent to the crack has opened up and admitted silver; peripherally adjacent inter-prismatic substance has become occluded by hypercalcification and shows a commencing translucent zone. In the other (left), near the dentine, inter-prismatic substance adjacent to the crack has opened up and admitted silver. Peripherally, the crack is occluded and adjacent inter-prismatic substance is hypercalcified and impermeable and shows a more advanced translucent zone than does the other (right) crack. In both cracks the adjacent dentine shows local reaction, though neither crack extends into it. × 125.

PLATE 35.

FIG. 10.—Young marsupial cheek tooth, permeated as a thick section from all sides by acid fuchsine; transverse section; shows a crack extending through the enamel and into dentine permeable throughout its extent. Photomicrograph in colour. × 105.

FIG. 12.—A portion of fig. 8, *c*. There are four cracks numbered 1, 2, 3, and 4.

1. This crack extends through the enamel and into the dentine. The dentine has reacted by forming a translucent zone of hypercalcification impermeable except centrally, where the tubes remain dilated and in direct continuity with the enamel crack, which is permeable throughout its length.
2. A crack extending through the enamel only, entirely impermeable to stain.
3. A crack extending through the enamel only; the peripheral portion is occluded.
4. A crack extending through the enamel only and permeable throughout.

Photomicrograph in colour. × 105.

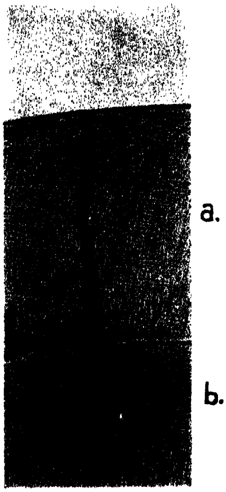
FIG. 13.—Young marsupial cheek tooth, permeated as a thick section from all aspects by acid fuchsine; transverse section; shows a crack extending through the enamel and into dentine; the dentine has reacted as in fig. 12, crack 1; the enamel crack is occluded peripherally. Photomicrograph in colour. × 100.

FIG. 14.—Young marsupial lower incisor, permeated from the pulp chamber by acid fuchsine: longitudinal section; shows a crack extending through the enamel and into dentine; the enamel crack is occluded, the translucent zone in the dentine is complete and no longer permeable centrally. Photomicrograph in colour. × 24.

FIG. 17.—Young marsupial lower incisor, from same section as fig. 14; shows an occluded crack in enamel. There are permeable translucent zones in both enamel and dentine. Photomicrograph in colour. × 24.

PLATE 36.

FIG. 19.—Young marsupial cheek tooth, permeated as a thick section from all aspects by acid fuchsine; transverse section; shows enamel crack permeable mesially and occluded peripherally as a linear translucency. × 70.



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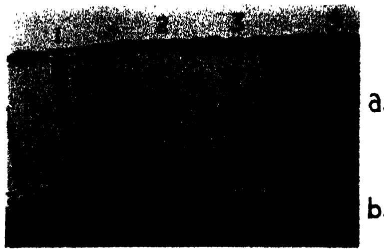

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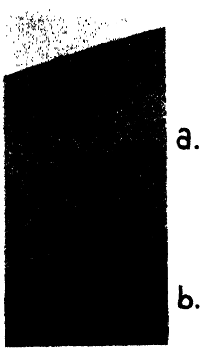

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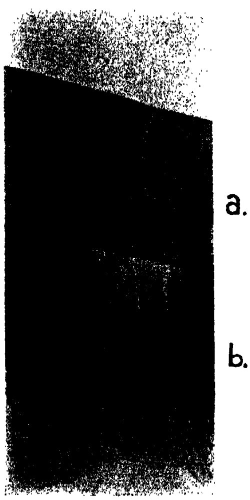

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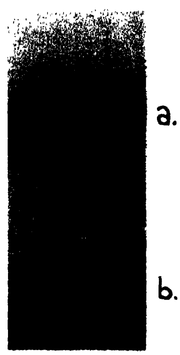

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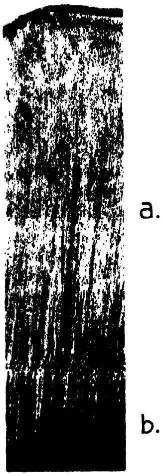

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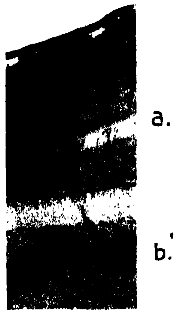

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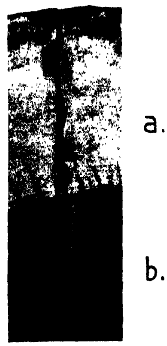

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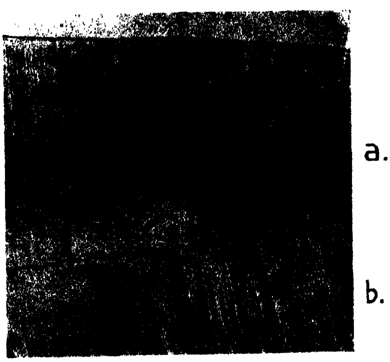

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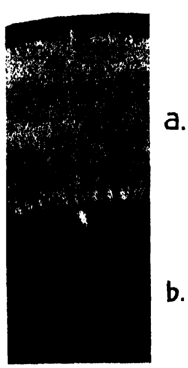

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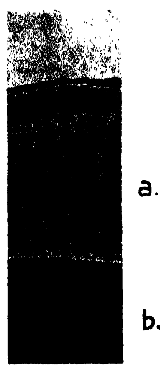

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Fig. 20.—A portion of fig. 8 shows occluded cracks in the enamel, some of which are in continuity with permeable cracks; the occlusions show up as linear hypercalcification. The enamel is here ground transverse to the long axis of its prisms. × 125.

Fig. 21.—Young human enamel from a transverse section of a permanent molar, permeated by 5 per cent. silver nitrate; metallic silver then being precipitated *in situ*; shows a crack extending through the enamel and into dentine, permeable throughout its extent; there is increased patency of dentine tubes at site of injury and opening up of inter-prismatic substance of enamel adjacent to the peripheral portion of the crack (compare with fig. 11). × 20.

Fig. 22.—Young human enamel from a transverse section of a premolar permeated with silver (as with fig. 21) after digestion with 1 per cent. trypsin; shows a crack extending through the enamel into dentine. The dentine has reacted by forming a translucent zone of hypercalcification, in which the central tubes are dilated and in direct continuity with the enamel crack, which is permeable throughout its length (compare with fig. 12, crack 1). × 22.

Fig. 23.—Human enamel from a longitudinal section of a maxillary permanent incisor permeated with silver, as with fig. 21; shows three cracks each extending through the enamel and into dentine. In each the peripheral portion of the enamel crack is occluded to a different degree, and there has been reaction in the dentine, as in fig. 22; also to a different degree in each case. (Compare with fig. 13.) × 45.

Fig. 24.—Young human enamel from a transverse section of a premolar permeated from the pulp chamber by methylene blue; shows a crack extending through the enamel and into dentine. The enamel crack is completely occluded and adjacent enamel hypercalcified; the dentine also has reacted and the translucent zone is no longer markedly permeable centrally. (Compare with figs. 14 and 17.) × 25.

Fig. 25.—Young human enamel from a transverse section of a permanent molar permeated from the pulp chamber by methylene blue; shows a crack extending through enamel only. It is permeable mesially and occluded peripherally as a linear translucency. (Compare with fig. 12, crack 3, and fig. 19.) The author finds that some linear translucencies are developmental in origin in human enamel. × 25.

The Production of Histamine from Histidine by Ultra-Violet Light and the Absorption Spectra of these Substances.

By R. B. Bourdillon, J. H. Gaddum, and R. G. C. Jenkins.

(Communicated by Dr. H. H. Dale, Sec.R.S.—Received April 10, 1930.)

(From the National Institute for Medical Research.)

It has been shown by Lewis and his co-workers (1927) that when the human skin is irritated by mechanical or chemical stimuli, by heat or cold, or by ultra-violet light, the triple response which occurs is due to the liberation of the so-called H-substance. Lewis considers that it is probable that this substance is a normal product of metabolism, the production or liberation of which is enhanced by various forms of irradiation. The chemical identification of the various substances liberated under these conditions is not possible, but they are found to possess so many physiological properties in common with histamine that there can be little doubt that this base is actually present among them.

It has been shown chemically that histamine can normally be obtained from extracts of liver and lung (Best, Dale, Dudley and Thorpe, 1927) and other tissues. Alcoholic extracts of most other tissues (Thorpe, 1928) including skin (Harris, 1927) also contain substances which possess the pharmacological properties of histamine and are almost certainly identical with it. Extracts made in other ways have similar properties, and there seems to be no reason to doubt that histamine is normally present as such, in varying quantities, in different tissues. Neither the source nor the ultimate fate of this histamine is definitely known, but it has been shown that the appearance of H-substance in the tissue spaces may be associated with the disappearance of histamine from the cells (Harris, 1927).

On the other hand, Ellinger (1928, 1929, 1930) has shown that a substance pharmacologically identified as histamine is produced when solutions of histidine are exposed to mercury-vapour lamps. He suggests that the erythema which occurs when skin is exposed to ultra-violet light is due to the direct conversion of histidine into histamine by the radiation. This theory is supported by evidence that the chemical change is produced almost entirely by radiation of those wave-lengths which are known to be mainly responsible for the production of erythema (310–280 $m\mu$). The considerable interest of the subject has led us to repeat some of Ellinger's experiments.

Methods.

The irradiations were carried out in small vessels of transparent fused silica with flat optically polished sides, 2·5 cm. square. The thickness of solution traversed by light was 1 cm. For the experiments of long duration, the histidine solutions were sterilised by heating to 100° C. after the cell was filled, and the long narrow inlet tubes were closed by plugs of cotton-wool or rubber teats. All histidine solutions were made up shortly before use, and portions were kept in the dark for comparison with the irradiated portions. These portions showed only very weak physiological action. The mercury-vapour lamp used was a K.B.B. quartz-mercury arc, working at atmospheric pressure and giving 2·5 amps. with 125 volts across the arc. The burner had been used for some hundred hours, but was not seriously "aged." The cell was 15 cms. from the lamp. The cobalt chloride filter consisted of a layer 1 cm. thick of 1 per cent. solution of cobalt chloride in alcohol *plus* 5 per cent. of concentrated hydrochloric acid, and was contained in a flat-sided silica cell with optically polished walls.

The absorption spectra were measured by a modification of the photographic method described by Dobson and Griffith (1927) as follows:--Light from a hydrogen discharge tube giving a continuous spectrum entered the slit of a Hilger spectrograph, after passing first through a cell containing the solution of the substance concerned, and then through a quartz rhomb designed to deflect on to the upper third of the slit only light which had passed through the centre of the cell. The lower two-thirds of the slit were covered by a rotating cam-shaped sector (Hilger), cut so that the time of exposure varied along the length of the slit. This variation was such that the logarithm of the time of exposure was proportional to the distance from a fixed reference line, and this sector thus replaced the wedge used by the above authors. The plates were developed in a narrow nickel tank with rapidly moving plunger to ensure uniformity (Dobson, Griffith and Harrison, 1926) and the density of the images was measured on an accurate photoelectric microphotometer (constructed for us by Dr. E. Schuster, to whom we are much indebted). This method merely involves a measurement for each wave-length of the distance separating two spots of equal density formed on the same plate during one exposure, and eliminates most of the common errors in photographic work.

The logarithmic sector was standardised against rotating V sectors, and thus Schwartzschild's constant was ignored, as is usual in absorption studies. A further check on the accuracy of the results was given by a careful measurement of the absorption of potassium nitrate; this was found to agree closely

with the composite curve published by Halban and Eisenbrand (1927). The time of exposure of solutions during photography was only 40 seconds, and since the hydrogen tube was a relatively feeble source of light, only negligible decomposition can have occurred during photography.

The physiological tests were carried out on cats anæsthetised with ether. The solutions were injected intravenously and the effect on the blood pressure recorded with a mercury manometer. Doses of the unknown solution and of a standard solution of histamine were given alternately, at a constant time interval of 3 minutes, until a match was obtained. The blood pressure was maintained as far as possible at a constant level by making occasional small adjustments in the depth of the anæsthesia. Part of a typical tracing (that for experiment 2) is shown in fig. 1. The drum was stopped after each effect had been recorded.

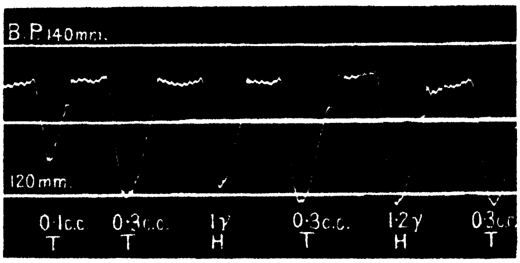

FIG. 1.—Blood pressure of cat under ether. Sensitivity increasing: 0·3 c.c. test solution (T) is more active than 1 γ (0·001 mg.) of histamine base (H) and about equivalent to 1·2 γ.

It will be seen that the injection of 1 γ (0·001 mg.) of histamine base [H] caused a smaller fall of blood pressure than that of 0·3 c.c. of the test solution T. On the other hand, 1·2 γ of histamine was about equivalent to this volume of the test solution. This method has been in use in this laboratory for some years for the assay of solutions containing histamine, and has been found to give results having an error of less than 20 per cent. Ellinger (1928) found that the effect of 1/100 mg. on the blood pressure of a cat under urethane was indistinguishable from that of 1/200 mg., and inferred that it was not possible to carry out accurate assays by this method. It seems probable that his failure to obtain a satisfactory measurement in this case was due to the use of doses that were too large for the purpose. Later (Ellinger, 1930) he found that the sensitivity of the cat varied so much that only gross differences in concentration could be detected with certainty. In our experience, with

cats under ether, these changes are seldom more rapid than that shown in fig. 1, where a slow increase in sensitivity was occurring. It is thus possible to get a reliable result, if the comparison is made only between the effect of any one injection and those of the injections given 3 minutes previously and 3 minutes later.

Results.

In the discussion which follows, the pharmacologically active substance produced by the irradiation of histidine is referred to as "histamine." No other substance having the same, or a similar, action is known to be produced from histidine, which readily yields histamine when appropriately treated with chemical reagents; but the identification was not chemically confirmed in these experiments.

The Absorption Spectrum of Histidine Dihydrochloride.

Ellinger (1929) found that the absorption of solutions of histidine showed a band indicating a maximum absorption of the rays, of wave-length about 280 $m\mu$, which are known to produce erythema, and which, according to Ellinger, are also particularly active in converting histidine to histamine.

Since this band was not seen under conditions similar to those of Ellinger's experiments, the observations were repeated using a 10 per cent. solution in a cell 1 cm. thick, thus giving an effective thickness 10 times that used by Ellinger. This experiment also showed no band. The results shown in fig. 2, curve A, are taken from measurements made with a 4 per cent. solution. For comparison fig. 3 shows portions of our curve and Ellinger's curve, plotted together on a much larger scale. The extinction coefficients on the left of the figures are those given by the formula

$$\varepsilon = \frac{\log_{10} I_0 - \log_{10} I_1 - K}{Cd},$$

where I_0 and I_1 are the intensities of light before and after traversing the solution, C is concentration in grams per litre, and d is the thickness of the solution in centimetres. K is the figure representing the absorption of the quartz cell filled with distilled water only, which must be subtracted from the total absorption in order to obtain the absorption due to dissolved substances.

On the right of the diagram are plotted extinction coefficients of the type used by Ellinger. These are equal to the above coefficients divided by $0 \cdot 1 \log_{10} e$. The curve shows no trace of the absorption band found by Ellinger, and remains almost flat until 250 $m\mu$, after which the absorption of shorter

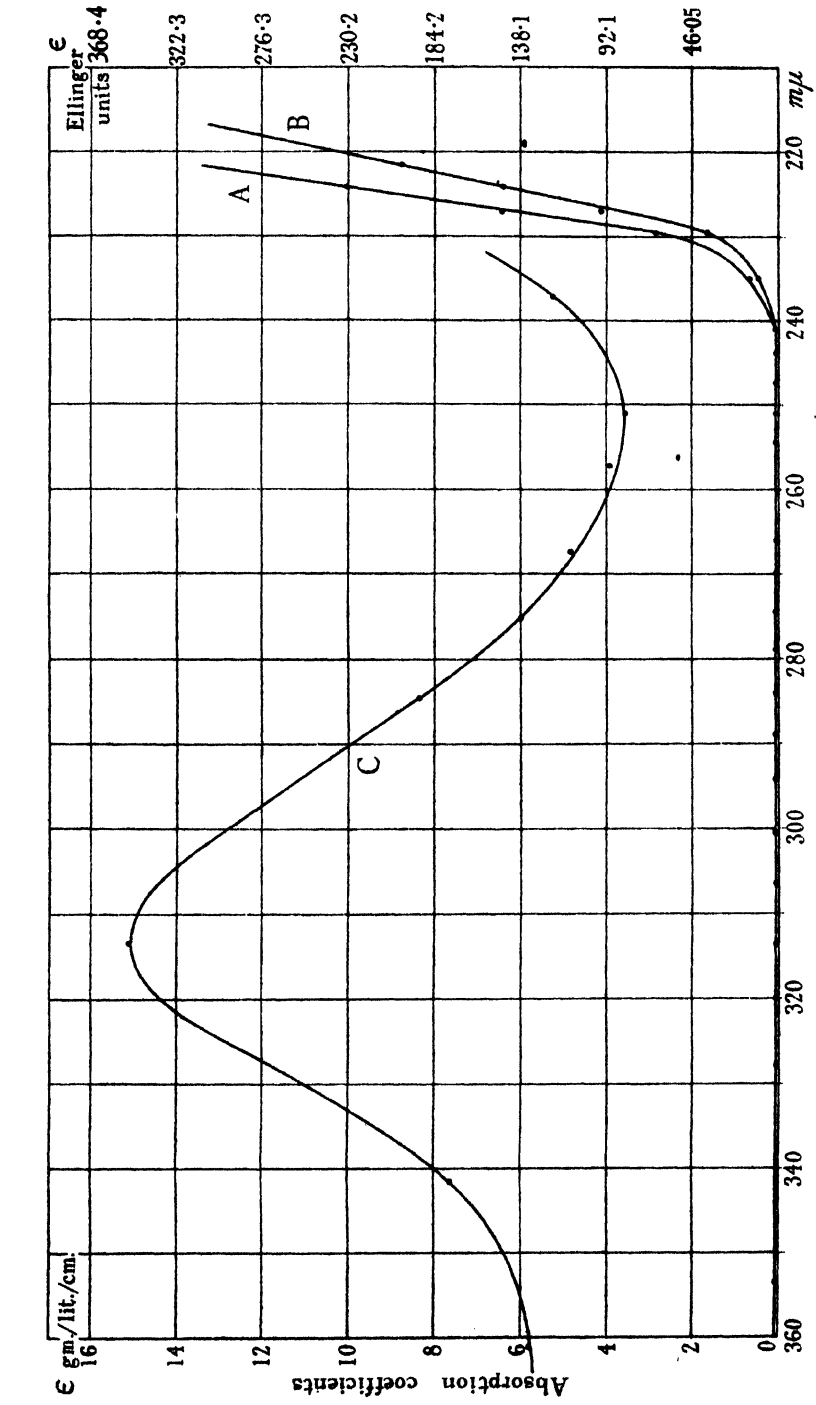

FIG. 2.—Absorption spectra of A, histidine dihydrochloride; B, histamine acid phosphate; C, histidine dihydrochloride after 15½ hours' irradiation.

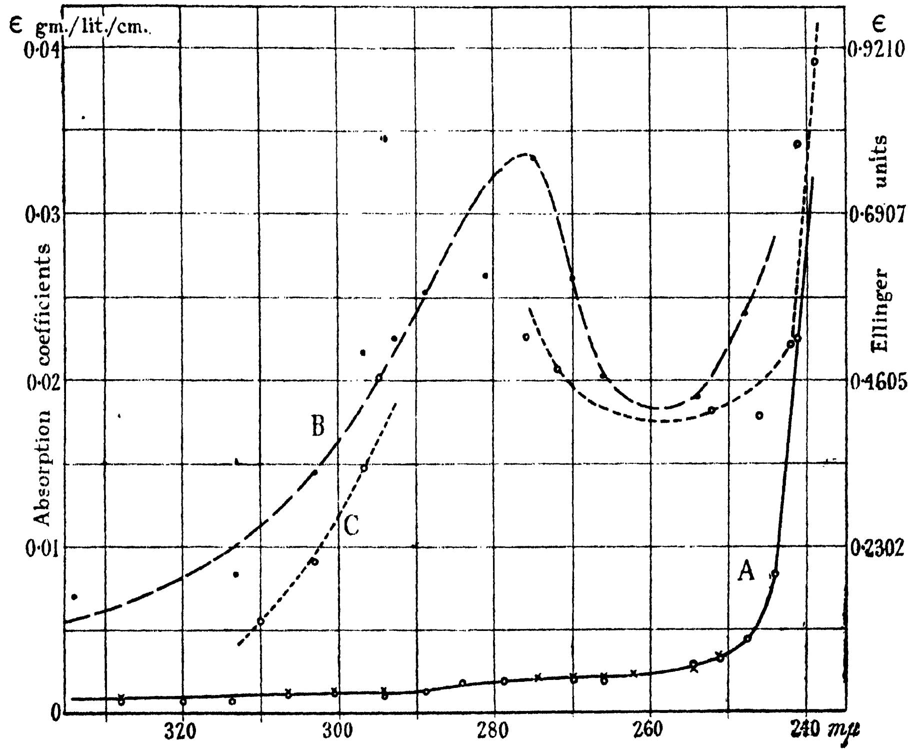

FIG. 3.—Absorption spectrum of histidine dihydrochloride. A, our results; B and C, Ellinger's results.

wave-lengths rises very rapidly. As the method is sensitive and accurate, we are forced to conclude that the absorption found by Ellinger was probably due to impurities in the histidine. Our own histidine was a very pure colourless sample of the dihydrochloride, prepared by Dr. H. W. Dudley, and kindly given to us by him.

The Absorption Spectrum of Histamine Phosphate in Water.

This is shown in fig. 2, curve B, and is closely similar to that of histidine, except that the sharp rise in absorption occurs at a wave-length about 2 $m\mu$ shorter for histamine than for histidine. The histamine phosphate was that sold by Messrs. Burroughs, Wellcome & Co., as ergamine acid phosphate.

Formation of Yellow Substance.

Our experiments agree with Ellinger's in showing the formation of one or more yellow substances on irradiation of histidine, and show that this colouration is caused by wave-lengths shorter than 265 $m\mu$.

The Absorption Spectrum of Irradiation Products of Histidine.

This is shown in fig. 2, curve C, which shows the ultra-violet absorption of a brown solution obtained by irradiating a histidine solution for 15½ hours with unfiltered light from a mercury arc. The curve shows an intense absorption band, with a maximum near 313 $m\mu$, which may be due to the yellow substance, or to some other component of the mixture. The absorption coefficients in this case were measured by a slightly different photographic method, using a high-tension spark as light source.

The Destruction of Histamine by Ultra-violet Radiation.

It is evident that tests of the production of histamine by radiation are liable to errors in interpretation unless correlated with similar tests of the destruction of histamine. Experiments 12 to 16 (Table II) confirmed Ellinger's observation that histamine is rapidly destroyed by the unfiltered radiation from a mercury arc, and showed that this destruction is chiefly due to wave-lengths shorter than 280 $m\mu$.

The Production of Histamine from Histidine by Irradiation.

(*a*) *By Unfiltered Radiation from a Mercury Arc.*—Table I, experiments 1, 2 and 3 show results closely resembling those obtained by Ellinger, namely, the formation of coloured solutions containing very small amounts of histamine which at first increase slowly, but after many hours' irradiation slowly decrease. The maximum quantity of histamine found was only of the order of 1/800 of the initial amount of histidine. The smallness of this quantity is probably due to the rapid destruction of histamine by the radiation used, and it is possible that a considerable fraction of the total histidine is converted to histamine, but that this is destroyed almost as fast as it is formed.

(*b*) *By Radiation of Selected Wave-lengths.*—It was observed by Ellinger that radiation from which wave-lengths shorter than 297 $m\mu$ had been excluded by filtration through "Nutglas," was as effective as unfiltered radiation in converting histidine to histamine. On the other hand, he was unable to produce histamine by radiation of wave-lengths 302 to 297, when this was obtained by the use of a very powerful monochromator. He was unable to explain this discrepancy, but believed that the experiment with Nutglas was quite correct, and quoted the production of histamine by these longer wave-lengths as evidence in favour of its production by sunlight, in which wave-lengths shorter than 295 $m\mu$ are not found in appreciable quantity.

Table I.

Experiment No.	Solution irradiated.	Source of light.	Filter used.	Wave-lengths of ultra-violet radiation reaching solution in appreciable quantity.	Duration of irradiation.	Resultant equivalent concentration of histamine base in mg./litre.	Colour of solution.
1	Histidine dihydrochloride 0·33 per cent.	Mercury arc	None	400–220	½ hour	1·25	Pale yellow
2	,, ,,	,,	,,	400–220	2 hours	4·0	Bright yellow
3	,, ,,	,,	,,	400–220	15½ hours	2·9	Brown
4	,, ,,	,,	Cobalt chloride in alcohol	400–265	,,	[0·22]*	Colourless
5	,, ,,	,,	Chlorine gas in 2·5 cm. bromine vapour in 5 cm. in separate cells	280–220	,,	2·0	Brownish yellow
6	,, ,,	,,	Vitaglass 2·3 mm. thick	400–290	,,	[0·0]	Colourless
7	Histidine dihydrochloride, but concentration 1 per cent.	Sun and sky light, March	None	400–295	Sun 2 hours, sky 12 hours, daylight	[0·0]	,,
8	,, ,,	Sun and sky light, May	,,	400–295	Sun 11 hours Sky 24 ,,	[0·0]	,,
9	,, ,,	,,	,,	400–295	Sun 41 ,, Sky 96 ,,	[0·2]	,,
10	,, ,,	Sun and sky light, June	,,	400–295	Sun 8 ,, Sky 48 ,,	[0·07]	,,

* The figures in brackets are those in which the activity was so small that the reading may have been seriously influenced by the error of measurement. As these observations were checked by repeated comparison of the weakly active irradiated solution with an unirradiated portion of the same solution, even the lowest finite figures indicate a real increase in histamine-like activity.

Table II.

Experiment No.	Solution irradiated.	Source of light.	Filter used.*	Wave-lengths of ultra-violet radiation reaching solution in appreciable quantity.	Duration of irradiation.	Resultant equivalent concentration of histamine base in mg./litre.	Colour of solution.
11	0·1 per cent. histamine phosphate, *i.e.*, 0·033 per cent. histamine base	Kept in dark	—	—	—	33·3	Colourless
12	,, ,,	Mercury arc	None	400–220	10 minutes	18	,,
13	,, ,,	,,	,,	400–220	1 hour	[0·0]*	,,
14	,, ,,	,,	Vita-glass as above	400–290	½ hour	26·6	,,
15	,, ,,	,,	,,	400–290	,,	30	,,
16	,, ,,	,,	Chlorine gas, bromine vapour as above	280–220	,,	18·7	Very pale yellow

* See note to Table I.

Our experiments with filters, however, agree with Ellinger's monochromator tests, and not with his filter experiments. Thus in experiment 6, using a Vita-glass filter cutting off wave-lengths shorter than 290 $m\mu$, we could detect no production of histamine ; and in experiment 4, using a cobalt chloride filter cutting off wave-lengths shorter than 265 $m\mu$, we only got a very small production of histamine. On the other hand, in experiment 5, using filters of chlorine and bromine which only transmitted wave-lengths shorter than 280 $m\mu$ (and some radiation of longer wave-lengths than 550 $m\mu$) we obtained histamine in a quantity of the same order as that obtained with unfiltered light. These experiments thus indicate that the wave-lengths shorter than 290 $m\mu$ are effective in producing histamine from histidine, while the longer wave-lengths are nearly or quite ineffective. This is what would be expected from the absorption spectrum of histidine, as shown in fig. 2.

Further tests were made with sunlight (experiments 7 to 10), so as to use a really intense source of the longer wave-lengths. These showed a very slow rate of formation of histamine $\cdot 10^{-5}$ mg. per square centimetre of exposed surface per hour or less. While it is not inconceivable that a similar rate of formation of histamine in the skin might produce an evanescent erythema, it is very improbable that sunlight, acting on the extremely minute quantities of free histidine presumably present in skin, can produce histamine at the same rate as when acting on a 1 per cent. solution of histidine in a quartz cell. Thus while the evidence available does not definitely disprove Ellinger's theory, our experiments with sunlight do not support it. The theory put forward by Lewis that the erythema occurring after exposure to ultra-violet light is due to the liberation of H-substance already present in the skin, or to disintegration products of killed cells, appears to us to provide a more probable explanation of the facts, especially since the greater part of the physiological reaction which follows exposure to ultra-violet radiation takes place long after the stimulus is removed.

Summary.

Experiments were made on the formation by the ultra-violet irradiation of histidine of a substance pharmacologically identified as histamine. The results confirm Ellinger's previous work in many respects, but differ in showing that wave-lengths shorter than 265 $m\mu$ are far more active in such formation than longer wave-lengths.

The ultra-violet absorption curves of pure histidine dichloride, and of histamine phosphate, have been measured, and found to show little absorption, except for wave-lengths shorter than 240 $m\mu$.

These facts do not support the suggestion that the erythema following irradiation of the skin is due to the formation of histamine from histidine by the simple chemical process which may be demonstrated outside the body.

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The Fluctuations of Bacterial Numbers and Nitrate Content of Field Soils.

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The changes in the numbers of bacteria and in biological activities in field soils at different seasons of the year have been studied by many workers. Hiltner and Stormer (1) found little difference between bacterial numbers in summer and winter. Remy (2) and Woitkiewicz (3) found highest bacterial numbers in spring, while Brown and Halversen (4) found two maxima, in February and in June. On the other hand Given and Willis (5) found highest numbers in September. Müntz and Gaudechon (6) and Lemmermann and Wichers (7) found nitrate production most active in spring, while Löhnis and Sabaschnikoff (8) found that urea decomposing, nitrifying and nitrogen fixing powers of soil were highest in spring and autumn, and Woitkiewicz (3) found that the nitrogen fixing and denitrifying powers of soil were highest in autumn. In spite of conflicting results, therefore, there is general evidence of increased bacterial numbers and activities in spring and in autumn. Russell and Appleyard (9), however, made bacterial counts and estimations of nitrate and CO_2 from soil every fortnight, and found that large fluctuations in bacterial numbers and activity occurred at these intervals.

A distinction between seasonal and more frequent changes in the micro-population was made by Cutler, Crump, and Sandon (10), who took samples from the dunged plot of Barnfield, Rothamsted, at daily intervals for a year and made counts of bacteria and of the encysted and active stages of two species of amœbæ and five species of flagellate. They found that in spring and autumn an increase in both bacterial and protozoal numbers took place, but that, superimposed on these seasonal changes, striking fluctuations both in bacteria and protozoa took place at very short intervals: the numbers on consecutive days often differing by over 100 per cent. More recently, a series of daily counts from lawn soil were made at Washington, by Smith and Worden (11). Their data when re-examined by Thornton and Fisher (12) were shown to indicate similar significant diurnal fluctuations in the bacterial numbers.

These results of diurnal samplings at Rothamsted and Washington made it

desirable to ascertain whether the micro-population and its activities in the soil changed at intervals of less than 24 hours.

The investigation was commenced on the same plot used by Cutler, Crump and Sandon, since on this plot the soil heterogeneity was known to be small. The method of sampling was as follows. The experimental plot was divided into four quarters and each sample was composed of a mixture of four cores removed to a depth of 6 inches with a sampling cylinder, successive samples being taken at 6-inch intervals. Samples after being passed through a 3-mm. sieve, were brought into the bacteriological laboratory and dilution platings made on Thornton's mannite agar (13). Owing to the number of counts made, it was not possible for all plates to be poured by one worker. Tests were therefore made to see if there was any variation in the technique of the workers employed. A sample of sifted soil was divided into four portions and platings prepared from each portion by a different worker. The results of two such tests showed that the technique of the different workers could not be distinguished.

It is known that the bacterial numbers alter fairly rapidly in soil brought into the laboratory. Any considerable variation in the time elapsing between taking the sample and pouring the plates might thus introduce variations in the count. To exclude errors from this cause the time taken in dealing with the soil and pouring the plates was carefully standardised and did not vary by more than 10 minutes. It was found by experiment that no significant change in bacterial content occurred in 30 minutes' storage.

It was also necessary to exclude the effect of uneven distribution of bacterial numbers over the plot, before changes in the count could be attributed to a fluctuating population. Four samples were therefore taken simultaneously from the plot in the manner actually employed for the 2-hourly sampling, each sample being a composite of four cores taken 6 inches away from the next sample. Platings were prepared from each sample by a different worker. The differences in count between the samples were quite insignificant, being wholly explained by the variance between the parallel plates, due to random distribution of the bacteria in the dilution fluid. The data from these tests were published by Thornton, Fisher and Mackenzie (14). Nitrate estimates were also made from each sample by Mr. H. J. Page and his assistants using the Devarda alloy method.

The first series of 2-hourly samplings were taken in July, 1921. The season had been exceptionally dry and no rain fell during the experiment or for some days previous to it. The sampling was carried out at 2-hourly intervals for

60 consecutive hours, individual workers operating in 8-hour shifts. The results of this experiment are shown in fig. 1. They indicate the existence of

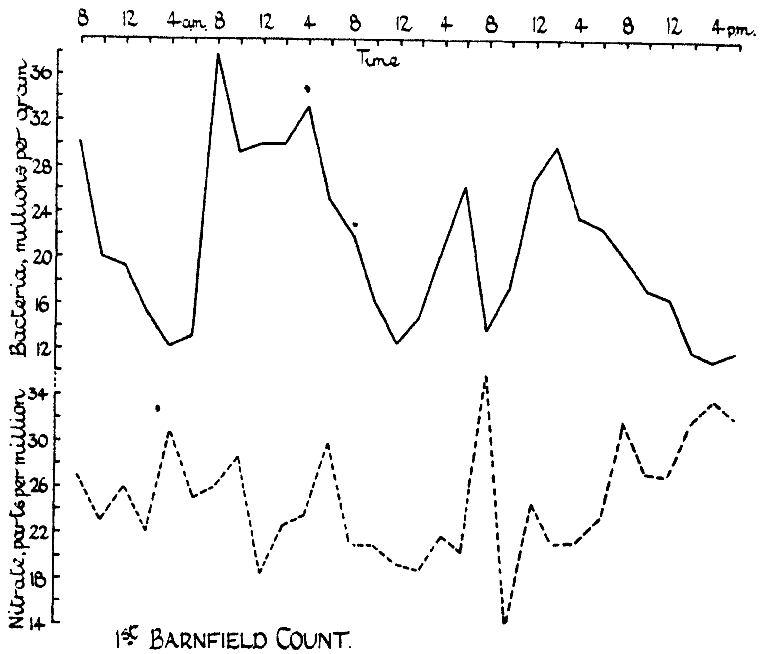


FIG. 1.

very striking fluctuations in bacterial numbers at short intervals and of changes in nitrate content.

A second experiment was made on the same plot in August, 1921, when 2-hourly samplings were carried out for 84 hours. Similar weather conditions prevailed. The results of this series confirm those of the first experiment (fig. 2). The fluctuations in bacterial numbers, though of lesser amplitude, are still large, and far outside the error of random sampling.

To ascertain whether similar fluctuations occurred in other localities having different soil conditions an experiment was planned at Kingsthorpe Hall, Northampton, in a field under barley stubble, having a light soil overlying Northamptonshire ironstone. Before carrying out this trial a plot was cleared, dug over to a depth of 8 inches and allowed to rest carefully weeded for 10 months. Before the trial was made, two series of simultaneous composite samples, 6 inches apart, were taken, as described in the case of the Barnfield plot, and, from these, bacterial numbers showed a standard error of 2·15 millions per gram from the first set of four samples and of 1·49 from the second

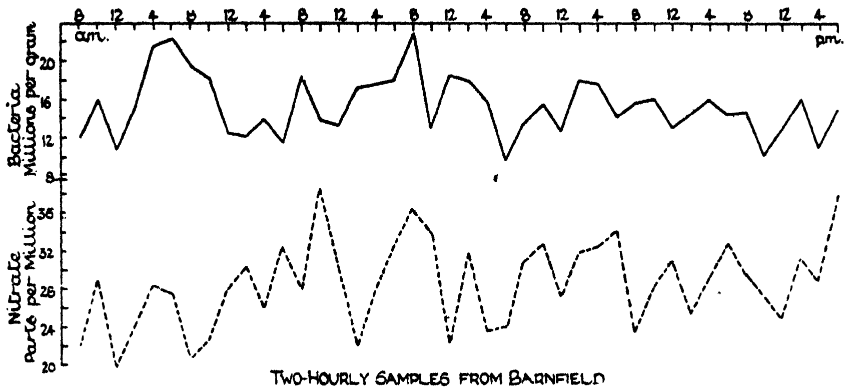


Fig. 2.

set. A series of 2-hourly samplings was made extending over a period of 80 hours. The bacterial counts from these samples are plotted in fig. 3. It is evident that, owing to the low bacterial numbers and their uneven distribution over the plot, the changes in count are not significant, with the exception of

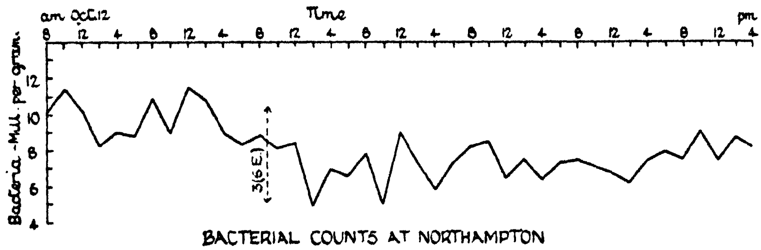


Fig. 3.

the fall from midnight on October 12 till 2 p.m. on the following day. This experiment shows the need for careful tests of variation over a plot, before the existence of changes in bacterial numbers and nitrate therein can be established. The uniformity found to exist over the Barnfield plot (14) is probably due to its continuous cropping with roots for a great number of years.

In order to remove as far as possible the factor of soil heterogeneity and to control more closely the conditions of the experiment, it was decided to prepare a special plot in the garden soil adjacent to Rothamsted Laboratory. Over an area of 6 by 12 feet the soil was removed to a depth of 9 inches, screened to remove stones, thoroughly mixed, and replaced in layers 2 inches deep, each layer being rolled before the next was laid down. The plot was then allowed to rest for 10 months. Before starting the sampling, a wood framework was erected over the plot and a tarpaulin sheet so arranged that the plot could be

entirely covered to exclude rain during the period of each series of samplings. Before the commencement of the 2-hourly sampling the variation in bacterial content over the plot was tested by taking simultaneous samples. The plot was divided into eight squares, and from each square four single-core samples were taken at 6-inch intervals with a sampling cylinder. From these bacterial numbers were estimated. Four workers took each set of four samples and poured the plates simultaneously. In this way the variation in bacterial content of the soil at 6-inch intervals in each portion of the plot was determined. The results are shown in Table I. The counts from three samples

Table I.—Distribution of Bacterial Numbers in eight sets of four single-core samples of Special Plot.

Time and date of sampling.	Sample No.	Colony count, mean of six plates	χ^2 index of dispersion between parallel plates	Means of four single-core samples and their standard errors
11 a.m., July 24, 1923	1	31·0	2·97	32·5±2·18 (excluding sample 3)
	2	29·7	2·74	
	3	(*56·7*)	(*12·04*)	
	4	36·8	1·81	
12 noon, July 24, 1923	5	41·8	5·28	41·4±0·71
	6	40·0	1·75	
	7	43·2	2·85	
	8	40·6	1·31	
3 p.m., July 24, 1923	9	36·5	3·77	42·4±2·70
	10	48·2	2·59	
	11	45·8	3·16	
	12	39·0	3·90	
5 p.m., July 24, 1923	13	43·6	1·31	39·6±1·68
	14	41·2	3·95	
	15	35·5	4·72	
	16	38·0	1·26	
11 a.m., July 25, 1923	17	(*53·7*)	(*27·83*)	66·7±2·58 (excluding sample 17)
	18	71·2	5·97	
	19	63·2	6·38	
	20	65·8	3·45	
12 noon, July 25, 1923	21	(*33·0*)	(*24·06*)	32·43±0·94 (excluding sample 21)
	22	30·7	2·09	
	23	33·8	2·45	
	24	32·8	5·20	
2 p.m., July 25, 1923	25	59·5	1·57	55·2±2·39
	26	50·0	1·64	
	27	52·3	1·13	
	28	59·0	6·92	
3 p.m., July 25, 1923	29	70·5	2·97	67·6±1·11
	30	68·7	3·78	
	31	66·6	3·20	
	32	64·5	1·57	

placed in brackets were excluded from consideration as the parallel plates disagreed. For the remaining 29 cores the standard error was 3·725 for a single core.

During the actual 2-hourly samplings, each count was made from a mixture of four cores. By doing this the effect of the sampling error is reduced, the standard error for the mean of four cores being 1·862 in the test above described, giving a percentage standard error of 4·73. In order to exclude the possibility of changes in the count being due to sampling errors, separate four-core samples were taken on each occasion from two halves of the plot, and only where similar changes in the count occurred in both halves of the plot were these regarded as representing true fluctuations in the bacterial population. Fig. 4 shows the method of sampling the plot during the experiments. At the first time of sampling bacterial counts and nitrate estimations were made from a mixture of cores from squares A1, B1, C1 and D1 and also from a mixture

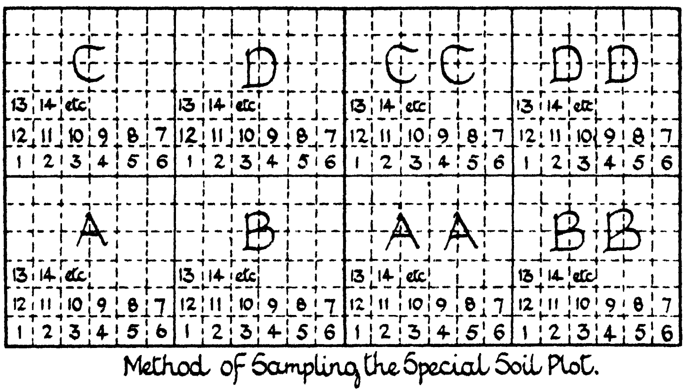


FIG. 4.

from squares AA1, BB1, CC1 and DD1. The second pair of samples were taken from A2–B2–C2–D2 and from AA2–BB2–CC2–DD2, each core being taken at a distance of 6 inches from the corresponding previous one. On days during which samples were taken the plot was kept protected from rain by mean of the tarpaulin roof. This was removed at other times in order to expose the plot to normal weather conditions.

Series of 2-hourly samplings were taken on August 10, 14, 17 and September 6, 1923, and on June 26, July 3, 16 and November 21, 1924, samples being taken at six or seven times on each date. The results from these experiments are shown in figs. 5 to 12, in which the data from the western half of the plot are shown with unbroken, and those from the eastern half by broken, lines.

Soil samples taken from a plot at different times must necessarily be taken also from different places. It is therefore necessary to show that the changes in bacterial numbers found at different times are not due merely to uneven distribution of the bacteria in space over the plot, before the existence of fluctuations of the population in time can be established. It must in fact be shown that the variation in numbers taken at different times significantly exceeds that between simultaneous samples.

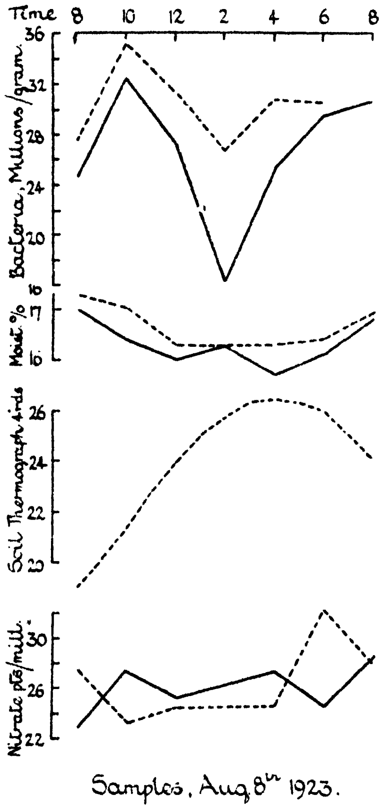


FIG. 5.

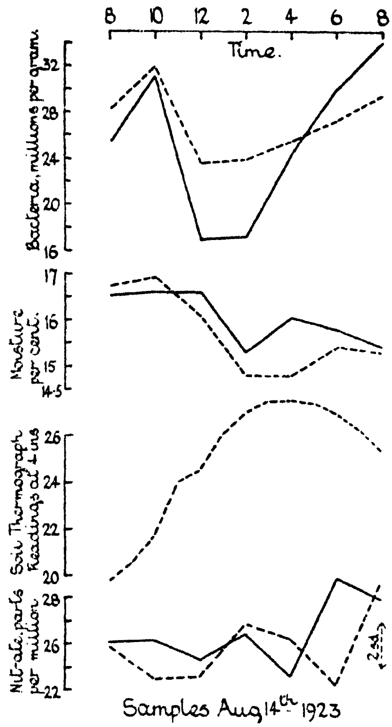


FIG. 6.

The series of samples illustrated in Table 1 shows that a change in numbers of 14·19 per cent. significantly exceeds the standard error of simultaneous samples from the experimental plot. In the sets of 2-hourly samples this range is often greatly exceeded by the fluctuations during the day.

Moreover, the existence of similar changes in the two halves of the plot shows that these cannot be due to the uneven distribution of the bacteria in space. The general agreement between the fluctuations in the two half-plots

is apparent in the curves shown in figs. 5 to 12. It is possible to measure this agreement and to test its significance by comparing the variance between mean bacterial numbers at different times with the variance between numbers from simultaneous samples. The method of making such an analysis of variance was developed by Fisher (15). His formulæ are given in terms of the deviations of class means from the general mean and of individual values from the class means. The inter-class variance, v_1, in this case between mean

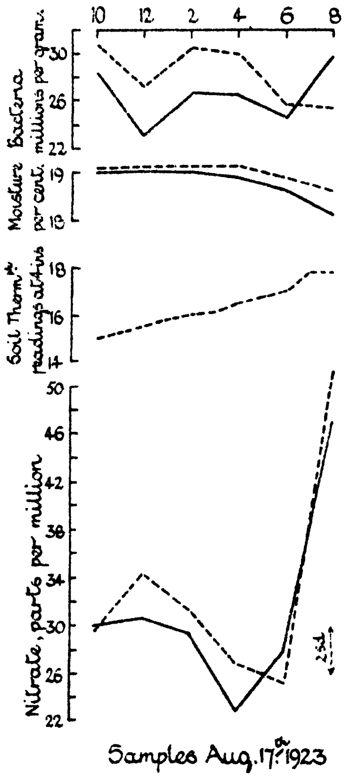


Fig. 7.

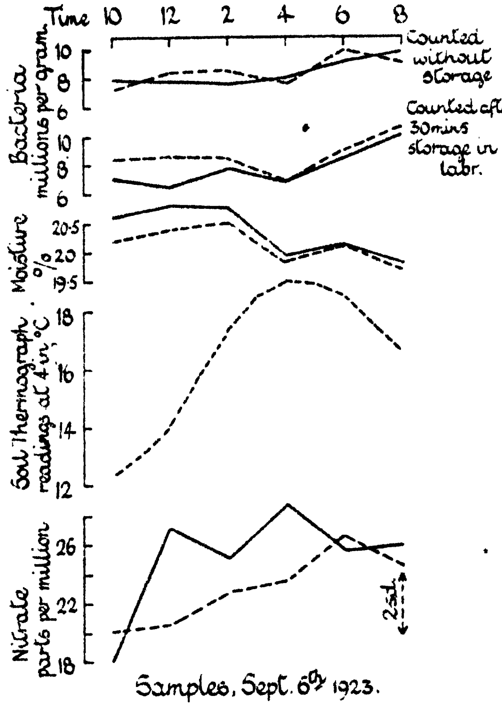


Fig. 8.

counts made at different times was calculated as shown below, the arithmetic being much simplified by the use of the last alternative formula.

$$v_1 = \frac{k \overset{n'}{\underset{1}{\mathrm{S}}} (\overline{x}_t - \overline{x})^2}{n' - 1} = \frac{k \overset{n'}{\underset{1}{\mathrm{S}}} (\overline{x}_t)^2 - k n' \overline{x}^2}{n' - 1}$$

$$= \frac{\overset{n'}{\underset{1}{\mathrm{S}}} (k\overline{x}_t \,.\, \overline{x}_t) - kn'\overline{x} \,.\, \overline{x}}{n' - 1} = \frac{\overset{n'}{\underset{1}{\mathrm{S}}} (\mathrm{T}_t \,.\, \overline{x}_t) - \mathrm{T}\overline{x}}{n' - 1},$$

where k simultaneous samples were taken on n' occasions and where T and $\bar{x}$ are the total and mean of all values and T_t and $\bar{x}_t$ the total and mean of each set or simultaneous counts. The variance v_2 between simultaneous samples

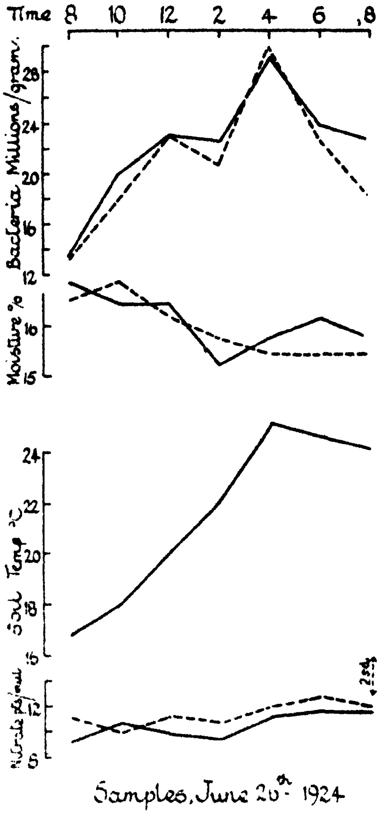


Fig. 9.

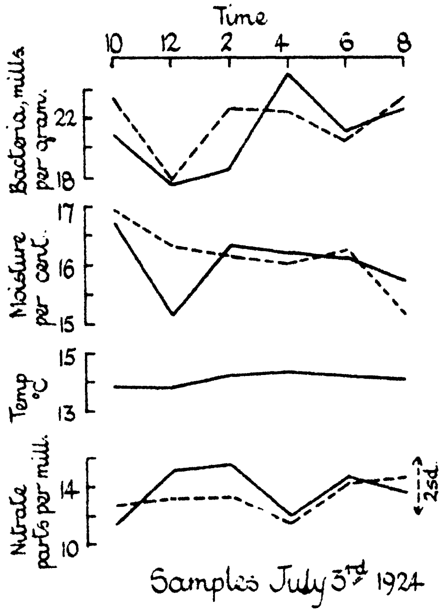


Fig. 10.

was obtained as follows, the alternative formula again simplifying the calculation,

$$v_2 = \frac{\overset{kn'}{\underset{1}{\mathrm{S}}} (x - \bar{x}_t)^2}{n'(k-1)} = \frac{\overset{kn'}{\underset{1}{\mathrm{S}}} (x)^2 - \overset{n'}{\underset{1}{\mathrm{S}}} (k\bar{x}_t \,.\, \bar{x}_t)}{n'(k-1)}$$

$$= \frac{\overset{kn'}{\underset{1}{\mathrm{S}}} (x)^2 - \overset{n'}{\underset{1}{\mathrm{S}}} (\mathrm{T}_t \,.\, \bar{x}_t)}{n'(k-1)},$$

where x is an individual value. In some experiments, notably that on 10th June, 1923, the bacterial numbers in the half-plots differed considerably; but the two populations, though of different sizes, showed similar fluctuations. When it

occurs, this difference in mean count between the half-plots increases the variance between simultaneous samples, but the value of the evidence based on the similarity in the fluctuations is not thereby diminished. It is therefore necessary to analyse the variance v_2 between simultaneous samples, into the variance v_3 between the mean numbers in the two half-plots, and the residual variance v_4 which results from uneven distribution of the bacteria within each

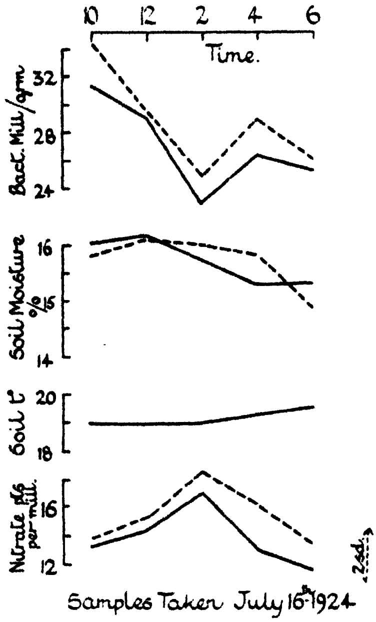


FIG. 11.

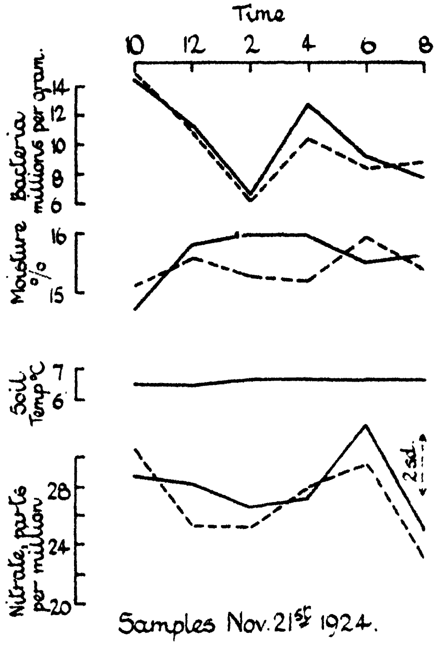


FIG. 12.

half-plot together with errors contained in the technique of counting. The inter-class variance v_3 was obtained from the formula

$$v_3 = \overset{2}{\underset{1}{\mathrm{S}}} (\mathrm{T}_p \,.\, \bar{x}_p) - \mathrm{T} \,.\, \bar{x},$$

and the residual variance v_4 by subtracting this sum of squares from that obtained for v_2, there being then one less degree of freedom representing the two half-plots.

$$v_4 = \frac{\left\{\overset{kn'}{\underset{1}{\mathrm{S}}} (\bar{x})^2 - \overset{n'}{\mathrm{S}} (\mathrm{T}_t \,.\, \bar{x}_t)\right\} - \left\{\overset{2}{\underset{1}{\mathrm{S}}} (\mathrm{T}_p \,.\, \bar{x}_p) - \mathrm{T} \,.\, \bar{x}\right\}}{(n-1)(k-1)},$$

where T_p and $\bar{x}_p$ are the totals and means of each half-plot. In the experi-

ments, $k = 2$, there have been two simultaneous samples, so that this formula reduces to

$$v_4 = \frac{\left\{\overset{2n'}{\underset{1}{S}}(\bar{x})^2 - \overset{n'}{\underset{1}{S}}(T_t \,.\, \bar{x}_t)\right\} - \left\{\overset{2}{\underset{1}{S}}(T_p \,.\, \bar{x}_p) - T \,.\, \bar{x}\right\}}{n-1}.$$

The statistic

$$z = \frac{\log_e v_1 - \log_e v_4}{2}$$

can then be applied to test whether the variance between samples taken at different times significantly exceeds the residual variance.

Table II shows the values of v_1, v_2, v_3 and v_4 given by the experiments. The

Table II.

Experiment	Time variance v_1.	Total variance between simultaneous samples v_2.	Variance between half-plots means v_3.	Residual variance v_4.	z test between v_1 and v_4.	$p = 0 \cdot 05$.	Correlation between fluctuations in the half-plots
10.8.23	547·85	205·80	903·07	66·34	1·0556	0·8097	0·784
14.8.23	708·82	151·50	135·16	154·22	0·7626	0·7274	0·643
17.8.23	111·16	95·35	164·28	81·56	0·1550	0·8097	0·153
6.9.23	23·16	4·17	14·16	1·68	1·3133	0·9272	0·865
26.6.24	874·02	47·86	117·81	33·87	1·6253	0·8097	0·925
3.7.24	143·80	31·31	12·20	35·13	0·7047	0·8097	0·607
16.7.24	346·35	57·92	119·03	42·64	1·0474	0·9272	0·781
21.11.24	272·53	5·69	7·05	5·42	1·9587	0·8097	0·961

Analysis of the variance in bacterial numbers from 2-hourly samples.

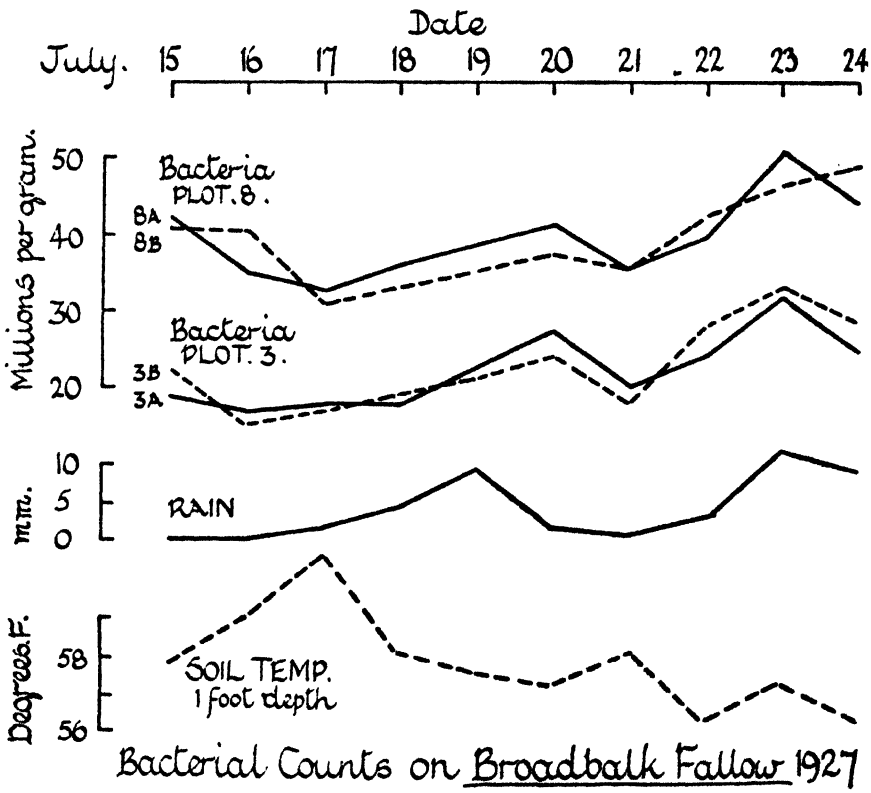
sixth column gives the value of z obtained by comparing v_1 with v_4, and the seventh column the value of z that would be exceeded once in twenty times by chance. The last column shows the correlation coefficients between the fluctuations in the two half-plots obtained from the formula :—

$$r = \frac{v_1 - v_4}{v_1 + v_4}.$$

The population changes at different times give a variance which significantly exceeds the residual variance in six experiments. In one experiment the significance is doubtful and in one the excess is insignificant. A result that could occur less than once in twenty times by chance has in fact been obtained in six out of eight experiments. The changes cannot therefore be due to uneven distribution of bacteria over the plot or to chance variation in technique,

but show that the bacterial population is subject to large fluctuations during the course of the day.

The results of some daily sampling on Broadbalk field, Rothamsted, made in 1927 on that portion of the field that was fallow, suggest that the fluctuations are confined to a fraction only of the bacterial flora. In this experiment samples were taken each day from Plot 3, unmanured, and from Plot 8, receiving 600 lbs. ammonium sulphate, 3·5 cwts. superphosphate, 200 lbs. sulphate of potash, 100 lbs. sulphate of soda and 100 lbs. sulphate of magnesia per acre. Each plot was sampled in a manner similar to that employed on the special plot described above, simultaneous samples being taken on each occasion from two areas about 6 feet apart. Fig. 13 shows the bacterial numbers from



Plots.	Correlation.
8A and 8B	0·81.
8A and 3A	0·86.
3A and 3B	0·88.
8B and 3A	0·75.
Bacteria and rain	0·88.

Fig. 13.

the two areas on each plot. The bacterial numbers on Plot 8 are approximately double those on plot 3, but the amplitude of the fluctuations on both plots is the same. This suggests that the size of the fluctuating population is approximately similar in the two plots the difference in numbers between the two plots being made up of non-fluctuating groups.

There is also some evidence that different groups of bacteria show different fluctuations. In the series of 2-hourly samples taken in July, 1921, an organism forming yellow colonies appeared on the plates. Fig. 14 shows the number of

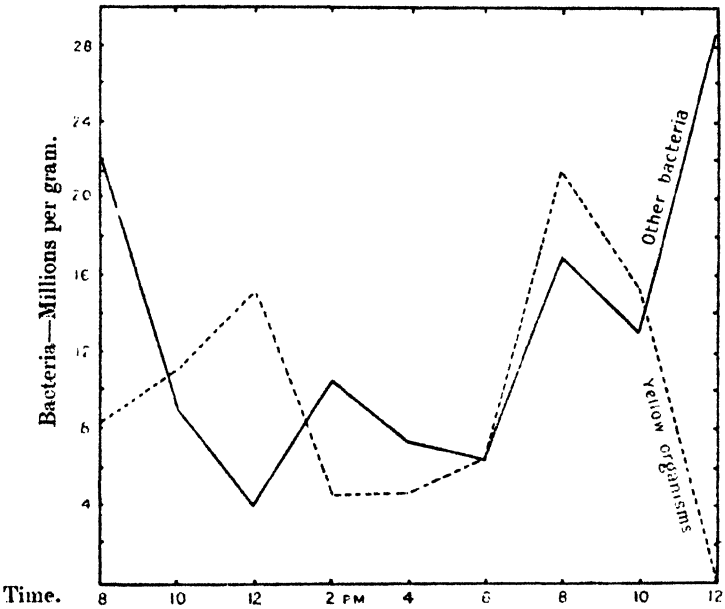


FIG. 14.—Fluctuating numbers of Yellow Organisms in Barnfield Soil, July, 1921.

this organism and the numbers of other organisms counted from 8 a.m. till midnight. The yellow organism shows fluctuations which differ from those of the remaining bacteria.

The daily counts made by Smith and Worden (11) from lawn soil show differences in fluctuation according to the medium used for plating. They took pairs or simultaneous samples each day and made platings of each sample on soil extract agar, on Waksman's egg albumen agar, and on the mannite salts agar that was used in the present work (Thornton, 13). The curves in fig. 15 plotted from their data, show the bacterial numbers plated on the latter two media, x and xx representing the two portions of the plot from which simultaneous samples were drawn. The fluctuations on these two portions show a positive correlation, as pointed out by Thornton and Fisher (12), but the bacteria

developing on the two media fluctuate differently. There is, for example, a big rise in numbers on September 6, which appears on the egg albumen media

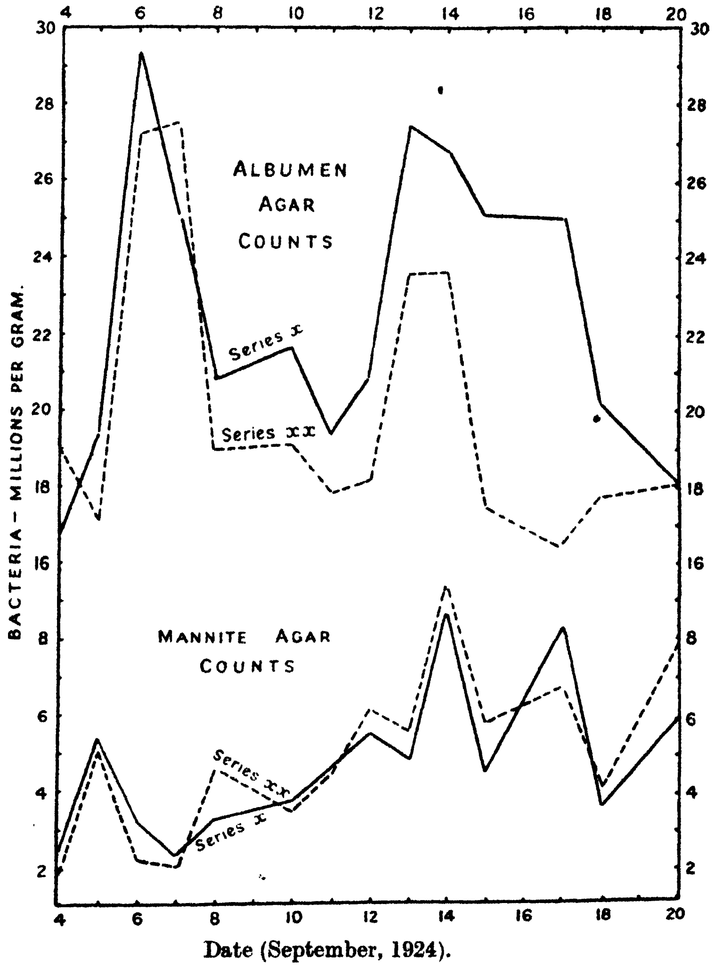


Fig. 15.—Fluctuating Bacterial Numbers in Lawn Soil at Washington. (Smith and Worden's Data.)

but not on the mannite salts medium. The soil extract medium allowed many more organisms to develop than the other two more differential media, and on this medium less significant changes were found. This also suggests that groups of bacteria showing different fluctuations are separated by the more differential media, but that on a generalised medium the fall in numbers on one group may be obscured by the increase of another group.

When considering the possible causes of the short interval fluctuations of

bacterial numbers in soil, the existence of a complex population of mutually reacting groups of micro-organisms must be borne in mind. The work of Cutler, Crump, and Sandon (10) showed that the fluctuations in bacterial numbers measured at daily intervals were inversely correlated with fluctuations in the numbers of active amœbæ. The laborious nature of the existing technique for counting protozoa makes it impracticable to count these at 2-hourly intervals. It is difficult, however, to account for the inverse relationship between bacteria and amœbæ found in daily soil samples unless it be assumed that it obtains in the case of the more rapid fluctuations through which the bacteria are known to pass.

There are three possible explanations of this relationship. The fluctuating numbers of amœbæ may be due to changes inherent in their reproductive cycle. Such changes would probably affect the numbers of bacteria upon which the amœbæ feed. Or the fluctuating numbers of the bacteria may be due to the changes inherent in their own reproductive cycle. They should affect the protozoal numbers, by producing alternate plenty and scarcity of food supply. Or, lastly, the fluctuating numbers may be due to outside causes such as changes in the physical environment.

The view that the protozoa are the cause of the bacterial fluctuations receives support from the experiment of Cutler (16), who compared the changes in bacterial numbers in sterilised soil inoculated with (*a*) bacteria alone, and (*b*) with bacteria plus protozoa, and found significant fluctuations in the latter case but not in the former. A mixture of only three species of bacteria was used, however, and their behaviour may not have been typical of those species comprising the population of a field soil. The present writer (17) has, indeed, found that *Bacillus radicicola* (Beij.), an organism common in arable soils, does show striking fluctuations in numbers when growing in pure culture in soil, and has attributed these fluctuations to the occurrence of a stage in the life cycle in which cocci are released from the mother cell. This suggests that the changes inherent in the multiplication rate of certain bacteria may be one of the primary causes of the fluctuations. The existence of different fluctuations in different groups of bacteria, if substantiated by future work, is most easily explained by this hypothesis.

The results of the daily counts from plot 3 and plot 8 of Broadbalk (fig. 13) indicate that under certain weather conditions rain may be a factor controlling the bacterial numbers. There is a close agreement between the fluctuations on the two plots, although these are about 50 yards apart and have wholly different soil conditions. The correlation between the counts from simultaneous

samples on the two plots was 0·845, while those between the counts from the two halves of the areas sampled were 0·88 for plot 3 and 0·81 for plot 8. This wide distribution of similar fluctuations can be explained by the fact that bacterial numbers show a correlation of 0·88 with rainfall. The weather during the course of this experiment was warm and there were several heavy showers. It seems, however, that only under special circumstances can rain control the short period fluctuations. The daily bacterial counts made on Barnfield by Cutler, Crump and Sandon (10) covering a year, show no correlation with rainfall, marked fluctuations occurring even during rainless periods. All the 2-hourly samplings, moreover, were made either during rainless periods or on a plot protected from rain. The factor of soil moisture is different from rainfall, which brings considerable amounts of dissolved oxygen into the soil. Neither the daily counts of Cutler, Crump and Sandon, nor the 1927 counts on Broadbalk, show any close agreement with soil moisture (fig. 16).

The 2-hourly counts from the special plot also show no correlation with soil moisture (figs. 5 to 12), marked changes in the count occurring for example on August 8, 1923 (fig. 5), when the soil moisture remained nearly constant.

Soil temperature also shows little correlation with the fluctuations in bacterial numbers. During the 2-hourly samplings made in 1924 the soil temperature at 4 inches was taken on each occasion of sampling and is shown in figs. 9 to 12. During three of the experiments, marked bacterial fluctuations occurred, although the soil temperature remained constant. On June 26 (fig. 9) however there was a big temperature wave in the soil, and this seems to be reflected in the bacterial count. During 1923 the temperatures were not taken from the plot itself, the curves given in figs. 5 to 8 being obtained from soil thermograph readings taken in another part of the grounds. Here also no correlation with bacterial numbers can be seen.

When the bacterial numbers obtained during the eight experiments on the special plot are compared, there appears to be some resemblance between the fluctuations in six of them. In fig. 17 the means of the counts obtained from the two halves of the plot are shown. On six dates there is a tendency for the count to be high at 10 a.m., low in the middle of the day and higher at 4 p.m. Of the two remaining experiments the bacterial numbers were very low and evidently abnormal on September 6, 1923, while on June 26 the big temperature wave may have been a disturbing factor (see fig. 9). Thus a definite periodicity in the fluctuations may normally occur, although a larger mass of data will be required to establish its existence.

Present evidence points, therefore, to a number of factors affecting bacterial

numbers in the soil, rain, soil temperature and protozoa being apparently able to modify fluctuations, a tendency to which may be inherent in the repro-

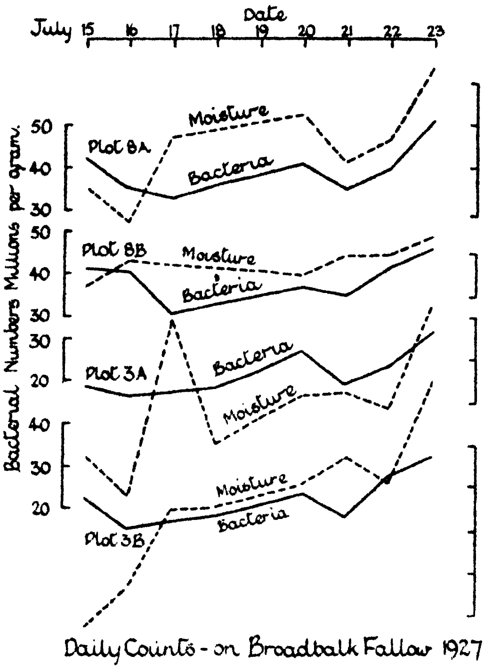


FIG. 16.

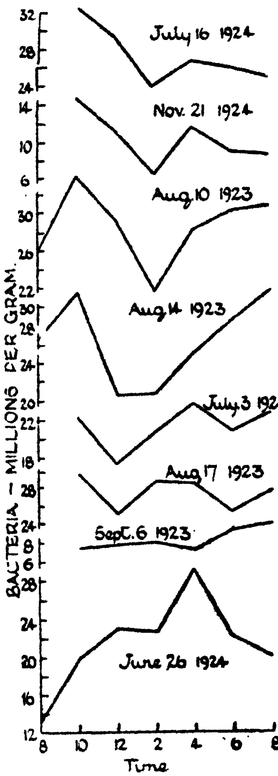


FIG. 17.

ductive cycles of a portion of the bacterial population. The proved existence of these rapid changes in the bacterial population must entirely modify our conception of the biological activities in the soil. It also profoundly affects existing methods for the microbiological analysis of soils, since scarcely any value can be attached to results obtained from single samplings.

The greater variation in nitrate content over the plot makes the existence of fluctuations more difficult to demonstrate. The parallel changes observed on July 16 and November 21, 1924, and especially on August 17, 1923, however, can scarcely be attributed to sampling error, and make the occurrence of true fluctuations highly probable. No correlation between nitrate content and bacterial numbers can be found. The existence of such rapid changes in bare soil protected from the leaching action of rain during the experiment

indicates the presence in the soil of micro-organisms which remove the nitrate at a rate sometimes exceeding its rate of production.

Summary.

Samples from a field soil were taken at 2-hourly intervals and from these bacterial numbers were counted by plating and nitrate estimated. Fluctuations in bacterial numbers greatly exceeding the variation in bacterial content of simultaneous samples were found to occur by day and night.

A special plot was prepared by screening the soil to remove stones and 2-hourly samples were taken from it, counts and nitrate determinations being made from two halves of the plot on each occasion of sampling. Fluctuations in bacterial numbers greatly exceeding the variation between simultaneous samples were found during the course of the day.

A comparison of the variance between samples taken at different times of the day and that between simultaneous samples showed that the results could not be due to uneven distribution of bacteria over the plot.

There is some evidence suggesting that different groups of bacteria show different fluctuations and that a portion of the population does not fluctuate.

No correlation between the changes in bacterial numbers and soil moisture content could be found. In one series of daily counts, the fluctuations were correlated with rainfall. Soil temperature changes have rarely shown any relation to the fluctuations.

Bacterial numbers have usually been high at 10 a.m. and low in the middle of the day. There is evidence suggesting that the nitrate content also fluctuates during the day.

Acknowledgments.

The authors gratefully acknowledge the assistance of Mr. D. W. Cutler and members of the Microbiology Department, who took part in making platings for the bacterial counts, also that of Mr. H. J. Page and members of the Chemistry Department, who carried out many of the actual samplings from the plots and made the estimations of soil nitrate.

They desire also to thank Dr. R. A. Fisher and Dr. J. Wishart for their advice and help in connection with the statistical analysis of the data.

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Cytochrome and Intracellular Oxidase.

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CONTENTS.

I. *Introduction.*

It was shown previously that intracellullar hæmatin compounds such as cytochrome or free hæmatin are very widely if not universally distributed in aerobic organisms and that the oxidation and reduction of cytochrome can be easily observed in intact living cells. It was also shown that in living cells oxidised cytochrome is reduced by organic molecules or metabolites activated by dehydrogenases, while the reduced cytochrome is rapidly oxidised by indophenol oxidase. Cytochrome acts in this case as a carrier between two kinds of respiratory enzymes of the cell: oxidases and dehydrogenases. This type of the respiratory mechanism of the cell is therefore composed of (1) dehydrogenases; (2) the organic molecules or metabolites; (3) the three components of cytochrome and the unbound hæmatin; (4) the indophenol oxidase; and (5) the molecular oxygen.

The main object of this paper is a more detailed study of the functional

relationship between the two components of this system: oxidase and cytochrome. For this purpose it was found important to reconstruct the oxidase-cytochrome portion of the system from its two components obtained separately from cells or tissues. Unfortunately the attempts to obtain an active oxidase preparation completely free from cytochrome have failed, and it was found impossible to extract all the three components of cytochrome from cells containing them. A great deal of information concerning the oxidase-cytochrome system can be obtained, however, by comparing the activity of the heart muscle oxidase preparation* with the somewhat reduced concentration of cytochrome, with the activity of the same preparation to which is added a certain amount of component *c* of cytochrome extracted from yeast cells. The activity of this system can be studied in relation to substances such as cysteine, the oxidation of which does not require the presence of a specific dehydrogenase. This study will enable us to compare the behaviour of oxidase and cytochrome in an artificially reconstructed system with their behaviour in living cells, and to determine with more precision the rôle they play in the processes of intracellular oxidation.

The main difficulty in this investigation is due to the fact that cysteine, as is well known (Warburg and Sakuma, 1923; Sakuma, 1923; Harrison, 1924; and Krebs, 1929), is so easily oxidised by traces of various metals. The latter may be present as impurities in the cysteine preparation itself, in buffer solutions, in the oxidase preparation, and in the preparation of cytochrome. Although cysteine and buffer solutions can be easily and to a great extent purified from metals, the preparations of oxidase and of cytochrome always contain a variable quantity of metallic impurities which are difficult to remove. It was found, however, during this study that with moderate precautions, even in the presence of the metallic impurities introduced by reagents, the activity of the oxidase-cytochrome system can be easily investigated.

As we shall deal here with only one type of oxidising enzyme, the indophenol oxidase of muscle, we shall refer to it in this paper simply as oxidase,

* The great advantage in using heart muscle is due to two main properties of this preparation, namely, (1) the oxidase on the surface of muscle particles, being exposed, can be brought into contact with cytochrome *c*; and (2) all the metabolites can be easily removed without damaging the oxidase. Yeast cells, on the contrary, cannot be as easily desaturated as muscle tissue, and in order to study the activity of the oxidase independently from that of other activating mechanisms we had to submit these cells to a higher temperature (52° C.) which has a much more destructive effect on dehydrogenases than on oxidase. In spite of these difficulties, it was found (1929) that the oxidase in yeast cells has the same properties and behaves in the same manner as the oxidase of the heart muscle preparation.

keeping in mind a few of its characteristic properties, namely, its reaction with intracellular hæmatin compounds, its oxidation of "Nadi" and other similar reagents, its insolubility in water and destruction by alcohol or drying. These properties distinguish it from polyphenol or catechol oxidase of plants with which it shares, however, the properties of being thermolabile, and inhibited by KCN, H_2S and CO (Keilin, 1929).

II. *Methods and Material.*

Buffer solutions were prepared from recrystallised phosphate salts dissolved in glass distilled water.

Cysteine.—Three samples of cysteine hydrochloride have been used as substrate for oxidation. Two of these samples, kindly given to me by Dr. M. Dixon, have shown a comparatively slow oxygen uptake in phosphate buffer solution of between *p*H 7·3 and 7·8. 8 mg. of one of them in 3 c.c. of buffer solution took up from 10 to 20 c.mm. of O_2 per hour, while the same amount of the second sample, twice recrystallised from metal-free HCl, took up only from 4 to 7 c.mm. of O_2 per hour. This sample of cysteine did not differ much from the metal-free cysteine obtained from Kahlbaum.

Oxidase Preparation.—The indophenol oxidase preparation is obtained from sheep's heart muscle by a method slightly different from that described in a previous paper (1929). Sheep's heart obtained directly from the slaughter-house is thoroughly cleansed from fat, ligaments and vessels. 55 g. of soft muscle is cut in fine pieces, washed twice, for 10 minutes each time, in 2 litres of tap water, squeezed through linen, mixed with 50 c.c. of clean sand (washed previously in concentrated HCl and distilled water) and pounded in a mortar to a fine paste. The paste is suspended in 400 c.c. of glass distilled water, rapidly separated from sand, and then centrifuged. The fluid is separated from the pulp and the latter detached from the remaining portion of the sand collected at the bottom of the centrifuge tubes. 38 g. of the pulp are uniformly suspended in distilled water or Ringer solution and made up to 190 c.c. One c.c. of this stock suspension contains approximately 200 mg. of the muscle pulp. 10 c.c. of the thick stock suspension is resuspended before each series of experiments in 50 c.c. of buffer or Ringer solution, 1 c.c. of which thus contains approximately 40 mg. of muscle pulp. The muscle suspension prepared in this manner has no oxygen uptake, shows hardly perceptible absorption bands of oxyhæmoglobin, and has a more or less reduced concentration of cytochrome, especially of its non-autoxidisable component *c*. Cytochrome seems to be removed from the exposed surfaces of the muscle particles and

there is some indication that the cytochrome remaining in the preparation is present within the particles of the suspension. The opalescent fluid obtained from the centrifuge tubes is more red in colour, contains more hæmoglobin than the pulp, shows absorption bands of cytochrome, especially those of the component *c*, and also contains the indophenol oxidase. This fluid does not change when filtered through the ordinary filter paper and on several occasions it was also used as oxidase preparation.

The main properties of the oxidase preparation obtained from sheep's heart-muscle have been described in the previous paper (1929, p. 222). The strength of the oxidase varies with the preparation and does not always correspond to the wet weight of the pulp, or even to the volume or weight of the precipitable proteins. Throughout this study, although the quantities of oxidase preparation used in every experiment were recorded, these quantities are significant only within the same series of experiments done with the same preparation, and as far as possible simultaneously. Another preparation of heart-muscle oxidase tested during this study was prepared from washed heart muscle pounded without sand. The fine suspension separated from coarse particles had in all respects the same properties as oxidase prepared with sand.

Component c of Cytochrome.—It was shown previously (1925, 1929) that of the three components of cytochrome, the component *c* is the most resistant to high temperature and to various reagents, and that it can be extracted with water from dry or acetone yeast. In extracts this component shows the same properties as in the living cells. More recently, in collaboration with R. Hill, this component was extracted from baker's yeast in a more purified and concentrated form. The simplest way of extraction is as follows. One kilogramme of fresh Delft baker's yeast is plasmolysed by mixing it with 50 g. of pure NaCl, and is rapidly poured into 3 litres of tap water boiling in a large (12 litres) enamelled vessel. The mixture is stirred for 5 minutes until the temperature reaches at least 90° C. and is then cooled rapidly by the addition of 6 kg. of broken ice. The suspension is left for 3 to 4 hours to settle, the supernatant cloudy greenish fluid is syphoned off, and the thick suspension of yeast left at the bottom is poured on to a large Buchner funnel covered with two layers of filter paper and a layer of kieselguhr. The fluid is sucked off by a vacuum pump and the yeast cake left on the funnel is washed by passing through it 100 c.c. or more of distilled water. The yeast cake (about 480 g.) is separated from the filter papers and mixed with 240 c.c. of distilled water, and 24 g. of sodium bisulphite. The mixture is stirred into a thick suspension, which is poured into a glass-stoppered bottle. After the addition of 24 g. of

finely powdered sodium hydrosulphite the mixture is thoroughly shaken in the bottle and left overnight. It is then poured into a large Buchner funnel covered with two layers of filter paper and a layer of kieselguhr. A pink, clear fluid is sucked off, the cake being washed with distilled water until it yields no more pigment. The pink fluid, which we shall call stock A of cytochrome, shows a strong absorption spectrum of the reduced component *c* of cytochrome. In addition to the pigment this solution contains a large amount of proteins and salts. After a thorough shaking with air and the addition of 0·7 per cent. of $CaCl_2$ a strong current of SO_2 is passed through the solution. A heavy precipitate of proteins with oxidised cytochrome *c* rapidly comes down. A portion of cytochrome which is still reduced remains in the solution, but on shaking the preparation with air and oxidising the pigment the latter gradually comes down with the remaining portion of the proteins.

The precipitate thus obtained is washed with distilled water which is either directly syphoned off or is removed by centrifuging. The washing is repeated several times until all traces of SO_2 and salts are removed from the preparation. The precipitate, which is of a greyish-brown colour, suspended in water and neutralised with NaOH gives a strong solution of cytochrome *c* of a red colour. This solution can be reprecipitated with SO_2, washed, and again dissolved. Once dissolved it remains soluble throughout the physiological range of *p*H. The strong solution of cytochrome *c* thus obtained is mostly in the oxidised state and shows an absorption spectrum of the parahæmatin type, mixed with faint bands of reduced cytochrome. On the addition of a reducer the colour becomes a pure pink, and the absorption spectrum changes from the parahæmatin to the hæmochromogen type. Although this cytochrome preparation contains a large amount of proteins it can be used in experiments with oxidase and cysteine.

On modifying slightly the method of preparation, however, it is possible to obtain a solution of cytochrome from which most of the proteins are removed. The manipulation remains the same until the pink fluid of stock A of cytochrome is obtained. After the addition of $CaCl_2$, instead of oxidising the pigment as we did previously, the pigment is kept reduced while the current of SO_2 is slowly passed through the solution. As soon as the proteins begin to aggregate and come down the current of SO_2 is shut off. The white protein precipitate, which is almost completely devoid of pigment, is separated from the fluid by filtration through a layer of kieselguhr. The pink fluid is then oxidised by shaking, and, after the addition of a small amount of $CaCl_2$, the current of SO_2 is passed once more. The fluid becomes turbid, and, on standing, a dark

brown precipitate composed of cytochrome with the remaining fraction of proteins slowly comes down. This precipitate, after being thoroughly washed with distilled water, is easily soluble at *p*H 6–9 giving a strong solution of cytochrome *c*. The separation of the first protein precipitate containing only traces of cytochrome was important in connection with this study and will be discussed later on.

III. *Properties of Component c of Cytochrome Extracted from Yeast.*

(1) Cytochrome *c* seems to correspond at least spectroscopically to the "modified myo- or histo-hæmatin" of MacMunn (1884–1887).

(2) A strong solution of oxidised cytochrome *c* is of deep red colour similar to that of 1 per cent. solution of alkaline methæmoglobin.

(3) In the oxidised state it has a typical absorption spectrum of a parahæmatin compound with two diffuse bands: α at 563 $\mu\mu$ and β at 528 $\mu\mu$, β-band darker and wider than α-band.

(4) When reduced it shows a very strong absorption spectrum of hæmochromogen type with α-band sharp and strong lying at 549·5 $\mu\mu$ and β-band which is not simple but appears to consist of three maxima the central of which, by far the strongest, is approximately at 520 $\mu\mu$.*

(5) When reduced, cytochrome *c* does not oxidise even on shaking the solution vigorously with air. Only after long standing in air it may gradually undergo a partial oxidation.

(6) It becomes autoxidisable at *p*H 4 on the acid side and *p*H 13 on the alkaline side, although it does not combine with CO. It reacts with CO only in a much more alkaline solution, forming a characteristic CO-cytochrome *c* compound, which is very sensitive to light, being rapidly dissociated when exposed even to the light of an ordinary filament lamp. In other words, cytochrome in strong alkaline solution, although its absorption spectrum remains unchanged, behaves like an ordinary hæmochromogen such as pyridine or nicotine hæmochromogen.

(7) In slightly acid or alkaline solution, for instance, alkaline phosphate buffer, cytochrome *c* is very stable and does not undergo destruction even if the solution is contaminated with bacteria. In this respect it differs from

* The complex structure of β-band of component *c* is mainly responsible for the structure of band *d* of reduced cytochrome as seen in living cells (insect muscle, for instance). The band β of the component *b* of cytochrome produces therefore only reinforcement of a portion of the complex band belonging to component *c*. If there is a β-band of the component *a* it would probably lie in the region of α-bands of *b* and *c*, and not in the region of band *d* as was suggested previously (1925).

ordinary hæmatin or hæmochromogen compounds such as pyridine hæmochromogen, which even on standing in test tubes exposed to air rapidly undergo modification and destruction.

(8) Reduced component *c* is oxidised neither by iron salts nor by autoxidisable hæmatin compounds. It is easily oxidised, however, by H_2O_2, by potassium ferricyanide and by copper salts.

(9) Reduced *c* is almost instantaneously oxidised when brought into contact with the oxidase of heart-muscle preparation.

(10) It seems that direct contact between the oxidase and cytochrome *c* is essential for this oxidation, as the reduced *c* when added to yeast suspension, fresh or warmed to 52° C. and cooled, which contains a strong oxidase, does not undergo oxidation even if shaken in air. Moreover, component *c* in boiled yeast cells does not oxidise on addition of heart-muscle oxidase.

(11) Oxidised *c* in solution is reduced by cysteine,* paraphenylenediamine, catechol, pyrogallol, etc., but not by compounds such as sodium succinate, sodium lactate, glucose, etc. Sodium succinate, however, reduces very rapidly the oxidised component *c* in the presence of muscle preparation, *i.e.*, when sodium succinate becomes activated by succin-dehydrogenase of the heart-muscle preparation.

(12) The oxidation of component *c* added to the muscle preparation is inhibited by small concentrations (0·001 M.) of KCN and H_2S, and irreversibly abolished by warming the muscle preparation to above 70° C.

(13) Reduction of component *c* by molecules (succinate, etc.) activated by dehydrogenases is delayed by narcotics and abolished by warming the muscle preparation to above 60° C.

(14) Unlike oxyhæmoglobin, but like hæmatin, parahæmatin or methæmoglobin compounds, oxidised cytochrome *c* is not reduced in vacuum. In other words, the reduction of cytochrome corresponds to a transition from ferric to ferrous compounds. The reduction of cytochrome within the cell, or of component *c* in presence of muscle preparation and a metabolite, is not directly due to a low oxygen tension, but to the presence of reducing substances, mainly the activated molecules of substrate.

(15) The peculiar properties of the component *c* of cytochrome are mainly due to the nitrogen compound which is united to the iron-porphyrin portion of its molecule.

* The oxidised cytochrome *c* of the muscle preparation itself, which is not exposed on the surfaces of the muscle particles, is not as easily reduced by cysteine as the solution of extracted cytochrome *c*.

All this shows that in the extract thus obtained the component *c* of cytochrome has the same properties as it has in intact living cells or in the extracts of dry or acetone yeast cells.

IV. *Oxidation of Cysteine by Oxidase Preparation and Cytochrome c.*

A drop of strong solution of reduced component *c* added to 3 c.c. of dilute heart-muscle preparation, which hardly shows its own cytochrome, becomes immediately oxidised. The oxidised component *c* can be reduced on the addition of a small amount of cysteine. The concentrations of oxidase, of *c* and of cysteine can be easily adjusted in such a way that, on shaking the test tube containing them, the component *c* becomes rapidly oxidised, while on standing it becomes reduced. The behaviour of component *c* in this mixture is therefore very similar to that of cytochrome in living yeast cells. This observation suggested several experiments which will be described presently. The first experiment was carried out in five differential Barcroft manometers. The left-hand flask of each manometer received 8 mg. of neutralised cysteine hydrochloride in 3 c.c. of M/8 phosphate buffer solution of *p*H 7·8. The right-hand flask of the first manometer received: 1·5 c.c. of dilute oxidase preparation (muscle suspension), 0·2 c.c. of cytochrome *c* solution, and 8 mg. of cysteine; of the second manometer: 1·5 c.c. of oxidase warmed to 80° C., 0·2 c.c. of cytochrome *c* and 8 mg. of cysteine; of the third manometer: 1·5 c.c. of oxidase and 8 mg. of cysteine; of the fourth manometer: 0·2 c.c. of cytochrome and 8 mg. of cysteine; and of the fifth manometer: 1·5 c.c. of oxidase and 0·2 c.c. of cytochrome. The contents of the right flasks were made up to 3 c.c. with phosphate buffer, giving the same concentration and *p*H as in the left flasks. Moreover, both left and right flasks in this and all the subsequent experiments received 0·3 c.c. of 5 per cent. KOH in the tubes fused inside the flasks. 1 c.c. of the muscle preparation corresponded to about 40 mg. of wet weight of tissue.

The concentration of cytochrome could only be estimated approximately by comparing it with standard solutions of pyridine meso-hæmochromogen, prepared from mesohæmatin,* the colour and absorption bands of which resemble somewhat those of the reduced cytochrome *c*. By this method it was found that 0·2 c.c. of cytochrome *c* used in this experiment correspond approximately to 0·015 mg. of hæmatin. The results of this experiment are

* The mesohæmatin compound which was kindly given to me by Mr. R. Hill was prepared by him according to the method of H. Fisher and B. Pützer ('Zeitschr. f. Physiol. Chem.,' vol. 154, p. 50 (1926)).

shown in fig. 1. A number of similar experiments have been repeated with different preparations of muscle oxidase, of cytochrome, and of cysteine containing variable amounts of metal impurities (experiments Nos. 1 to 6, Table I).

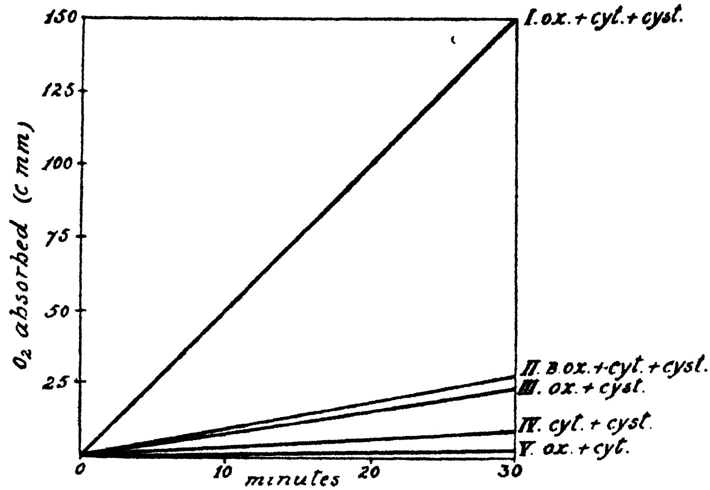

FIG. 1.—Oxygen uptake by : I, oxidase, cytochrome *c* and cysteine ; II, boiled oxidase, cytochrome and cysteine ; III, oxidase and cysteine ; IV, cytochrome and cysteine ; V, oxidase and cytochrome. (See experiment 1, Table I.)

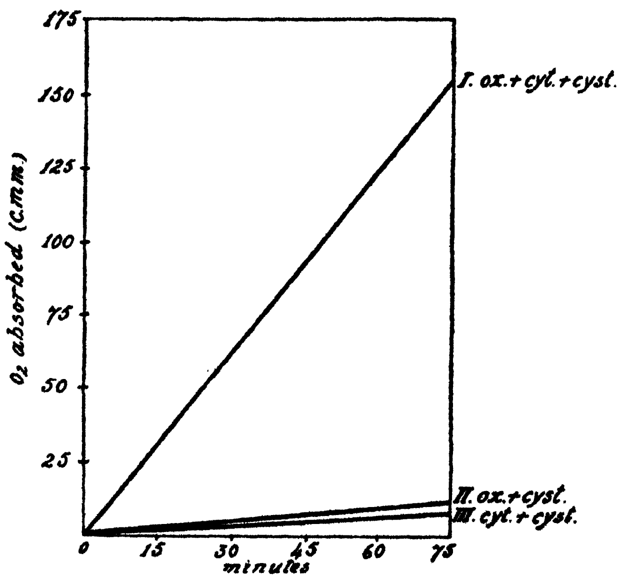

FIG. 2.—Oxygen uptake by : I, oxidase, cytochrome and cysteine ; II, oxidase and cysteine ; III, cytochrome and cysteine. Oxidase preparation obtained from heart-muscle cells washed and suspended in Ringer solution. (See experiment 11, Table I.)

The strength of the oxidase preparation varied naturally from one series of experiments to another, and the quantities of preparation used have therefore no absolute meaning. Other experiments have been carried out in a similar way except that the left-hand flasks instead of cysteine received 3 c.c. of buffer solution (experiments Nos. 7 to 9, Table I). All these experiments gave, however, very uniform results (figs. 1 and 2 and Table I), namely, that neither

Table I.—Oxidation of Cysteine by Oxidase-cytochrome System.

Experiment No.	t.	Time.	Muscle oxidase.	Cytochrome.	Cysteine.	O_2 uptake.
	° C.	mins.	c.c.	c.c.	mg.	c. mm.
1	21	33	1·5	0·2	8	166
			1·5	0	8	28
			0	0·2	8	11
			1·5B	0·2	8	30
			1·5	0·2	0	4
2	16	42	1·5	0·2	8	224
			1·5	0	8	35
			0	0·2	8	15
3	16	53	1·0	0·2	8	220
			1·0	0	8	38
			0	0·2	8	16
4	16	44	1·0	0·1	8	142
			1·0	0	8	40
			0	0·1	8	6
5	18	31	1·8	0·2	8	152
			1·8	0	8	33
			0	0·2	8	25
6	20	37	1·8	0·2	8	167
			1·8	0	8	40
			0	0·2	8	7
			1·8B	0·2	8	20
7	22	33	1·5	0·2	8	186
			1·5	0	8	34
			0	0·2	8	25
			0	0	8	5
8	22	35	1·5	0·2	8	215
			1·5	0	8	40
			0	0·2	8	20
9	17	40	1·5	0·1	8·5	135
			1·5	0	8·5	8
			0	0·1	8·5	6
10	20	23	1·8	0·5	8	195
			1·8	0	8	18
			0	0·5	8	7
			1·8	0·02	8	55
11	17	75	1·5	0·2	7·5	155
			1·5	0	7·5	12
			0	0·2	7·5	8

oxidase by itself, nor cytochrome *c* by itself, can appreciably oxidise cysteine, and that only when both oxidase and cytochrome are brought together cysteine undergoes a rapid oxidation. In the experiments shown in fig. 1 and experiments 1 to 6, Table I, the small oxygen uptake due to autoxidation of cysteine in buffer solutions was balanced by the presence of cysteine in the left-hand flask, while the oxygen uptake by oxidase and cytochrome *c* in the absence of cysteine was found to be almost negligible (curve V, fig. 1).

The small rate of oxygen uptake given by cysteine in the presence of cytochrome *c* alone was mainly due to the metallic impurities in the cytochrome preparation, while the slow uptake of oxygen by cysteine in the presence of the oxidase preparation alone was partly due to the metallic impurities and partly to the small amount of cytochrome present within the muscle particles which gradually comes into contact with the cysteine.* Moreover, boiling the oxidase preparation abolishes the activity of the oxidase-cytochrome system (fig. 1, curve II, and B of experiments 1 and 6, Table I).

Similar results can be obtained also with the oxidase prepared from unwashed heart-muscle, thoroughly pounded with sand and suspended in Ringer or buffer solution. The muscle suspension alone prepared in this way has a low oxygen uptake† and only slowly oxidises cysteine without the addition of cytochrome *c* in solution. One of the experiments carried out with such muscle preparation gave the following results :—

(Oxidase, 60 mg. muscle wet weight; cytochrome, 0·2 c.c.; cysteine, 10 mg.; time, 50 minutes in Ringer solution *p*H 7·6.)

Oxidase + cytochrome	Took up	22	c. mm.	of O_2.
Oxidase + cysteine................	,,	53	,,	,,
Cytochrome + cysteine	,,	8	,,	,,
Oxidase + cytochrome + cysteine ..	,,	210	,,	,,

It may be noted here that the oxidised cytochrome *c* of the muscle preparation itself is not as easily reduced by cysteine as the oxidised cytochrome *c* in solution. It is possible that a portion of the cytochrome of the muscle preparation, not being exposed on the surfaces, is not sufficiently accessible to cysteine.

* The muscle preparation, as will be demonstrated (p. 438) contains more metallic impurities than is shown in these experiments. Muscle and probably other tissue preparations have a great capacity for taking up and inactivating various catalytically active metals. This property of tissue preparations makes the above experiments with cysteine much easier to carry out.

† This low oxygen uptake is due to the destruction of some of the activating mechanisms of cells as well as to disappearance of the available supply of metabolites.

Moreover, the reduction of cytochrome by cysteine is not very rapid, and for the rate of oxidation of cysteine, which was generally obtained in our experiments, even autoxidisable hæmatin compounds would be required in a much higher concentration than that of cytochrome present in our dilute muscle preparation. It must be noted that during all these experiments the properties of cytochrome *c* remained unchanged.

V. *Rate of Oxidation of Cysteine at Different Concentrations of Oxidase and of Cytochrome.*

The rate of oxidation of cysteine depends on the concentration of oxidase as well as on that of cytochrome. This can be shown by measuring the oxygen uptake by cysteine in the presence of a definite amount of cytochrome and increasing concentrations of oxidase, or in the presence of a constant amount of oxidase and different concentrations of cytochrome. In the first series of experiments the right-hand flasks of the four differential manometers received cysteine, cytochrome and increasing concentrations of muscle preparation, while the left flasks received only the same amounts of cysteine with the corresponding amounts of muscle preparation. In the second series of experiments the right flasks received cysteine, muscle preparation and the increasing amounts of cytochrome, while the left flasks received cysteine and the corresponding quantities of cytochrome. The oxygen uptake due to cysteine and the various concentrations of oxidase alone, or of cytochrome alone, was therefore balanced on both sides of the manometers. The muscle preparation used in these experiments was very carefully washed with glass-distilled water. It contained less metallic impurities than the preparations used in previous experiments, but also less oxidase, as its activity was reduced during long washing. The results of these experiments are given in Tables II and III and figs. 3 and 4.

These experiments show that the velocity (V) of oxygen uptake of cysteine is not in direct linear proportion to the concentration of either oxidase (x) or cytochrome (y). Except for the low concentrations of oxidase the reaction can be expressed by the equation $V = \kappa x^{1/n}$ or $V = \kappa y^{1/n}$ where $n = 2$. In other words the velocity of oxidation of cysteine seems to follow approximately the so-called rule of Schutz, namely, that the rate of oxidation is proportional to the square-root of oxidase concentration when the concentration of cytochrome is constant, or to the square-root of cytochrome concentration when the concentration of oxidase remains constant. It is, however, impossible to say yet whether this rule will be proved general for the system, as the above results based on very few experiments may be due to some peculiar conditions common

Table II.—Effect of Oxidase Concentrations on the rate of Oxygen Uptake by 20 mg. of Cysteine in presence of 0·25 c.c. of Cytochrome *c* solution. Concentration 1 of oxidase corresponds to 30 mg. of wet weight of muscle (see fig. 3).

Experiment.	Oxidase concentrations x.	Rate of O_2 uptake c. mm. per $\frac{1}{2}$ hour V.	$K = \frac{V}{\sqrt{x}}$.
1	1	50	(50)
	2	118	83
	3·3	152	83
	6·6	186	73
2	1	42	(42)
	2	93	65·8
	4	130	65
	6·8	161	62·5

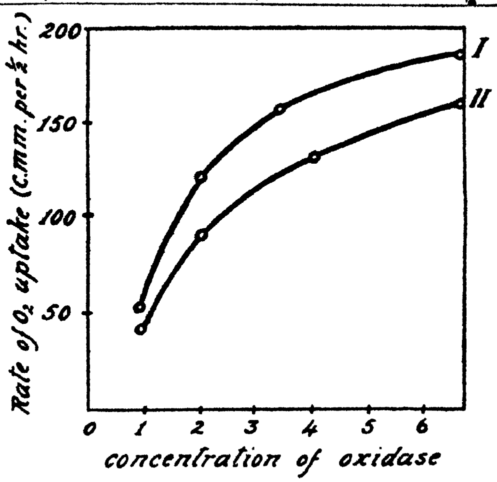

Fig. 3.—Rate of oxidation of cysteine in presence of constant concentration of cytochrome *c* and increasing concentrations of oxidase preparation. (See Table II.)

Table III.—Effect of Cytochrome Concentrations on the rate of Oxygen Uptake by 20 mg. of Cysteine in presence of 120 mg. of muscle preparation (oxidase). Concentration 1 corresponds approximately to 0·08 c.c. of cytochrome *c* preparation (see fig. 4).

Cytochrome *c* concentrations y.	Rate of O_2 uptake c. mm. per $\frac{1}{2}$ hour V.	$K = \frac{V}{\sqrt{y}}$.
1	62	62
3	120	69
6	170	69·5
12	218	63

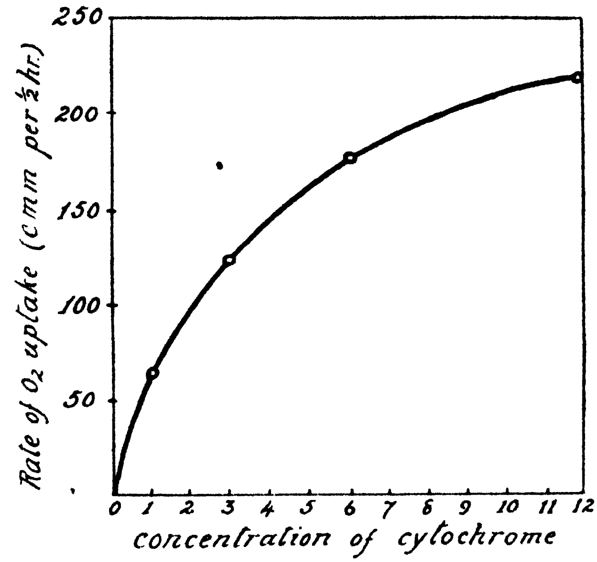

FIG. 4.—Rate of oxidation of cysteine in presence of constant concentration of oxidase and increasing concentrations of cytochrome *c*. (See Table III.)

only to these experiments. It was also found that in cases of low concentrations of oxidase the velocity of oxidation becomes proportional to the concentration of enzyme (curve I, fig. 5).

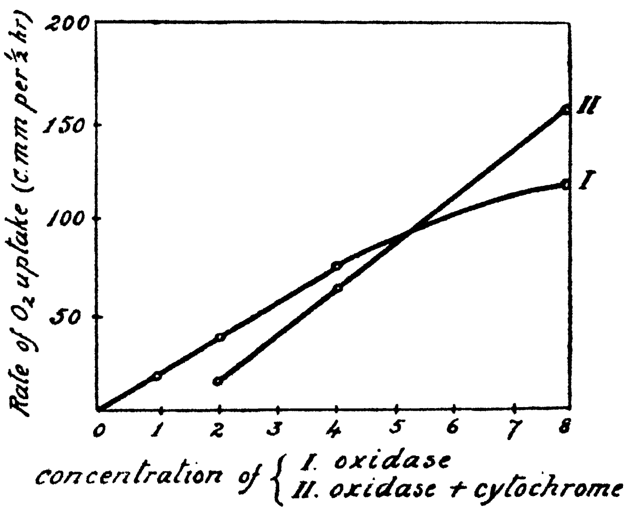

FIG. 5.—Rate of oxidation of cysteine in presence of: I, increasing concentrations of oxidase and constant concentration of cytochrome *c*. II, increasing concentrations of oxidase + cytochrome mixture. Concentration marked 4 for both curves corresponds to 52 mg. of wet weight of muscle and 0·18 c.c. of cytochrome *c*.

Different results are obtained when the concentrations of oxidase and of cytochrome are varied simultaneously. For this experiment a certain amount of cytochrome solution was added to the oxidase preparation. The right-hand flasks of the manometers received cysteine and different amounts of oxidase-cytochrome mixture, while the left-hand flasks received cysteine and corresponding amounts of oxidase preparation. The oxidation of cysteine due to the increasing concentrations of cytochrome alone was not balanced in this experiment, as the preparation of cytochrome being sufficiently free from metallic impurities hardly accelerated the oxidation of cysteine. At the lower concentrations of oxidase-cytochrome there is a distinct delay in the oxygen uptake by cysteine, so that only the maximum velocities corresponding to each concentration were compared with each other. The results of this experiment, represented by the curve II, fig. 5, show that except for the very low concentrations of catalytic system the rate of oxygen uptake by cysteine is a linear function of oxidase-cytochrome concentration.

The experiments described in this chapter show also that the concentration of cytochrome does not give directly the measure of the respiratory activity of the cell. This activity, as we have previously seen (1929), depends on the behaviour and concentrations of all its components, such as oxidase, cytochrome, dehydrogenases, metabolites and oxygen. Each of these components may become a limiting factor in the respiratory process of the cell.

The fact that in nature there is a marked parallelism between the distribution of cytochrome and the respiratory activity of cells shows that the conditions which are favourable for the formation of one component such as cytochrome are usually favourable for the development of the other components of this respiratory system, namely, oxidase and dehydrogenases.

VI. *Effects of* KCN, *of* H_2S *and of Ethyl-Urethane.*

One of the experiments on the effect of KCN, H_2S and narcotics was carried out in four differential manometers, the left-hand flasks of which received 3 c.c. of buffer solution, while the right flasks received 1·5 c.c. of oxidase preparation, 0·2 c.c. of cytochrome *c*, and 8 mg. of cysteine. In addition to these reagents, to the right flask of the second manometer was added 5 per cent. of ethyl urethane, to that of the third 0·001 M. of Na_2S, and of the fourth 0·001 M. of KCN, the contents of all the flasks being made up to 3 c.c. with buffer solution. The results of this and other experiments, which are given in fig. 6 and Table IV, show first of all that KCN in a low concentration inhibits completely the oxidation of cysteine by the oxidase-cytochrome system, while

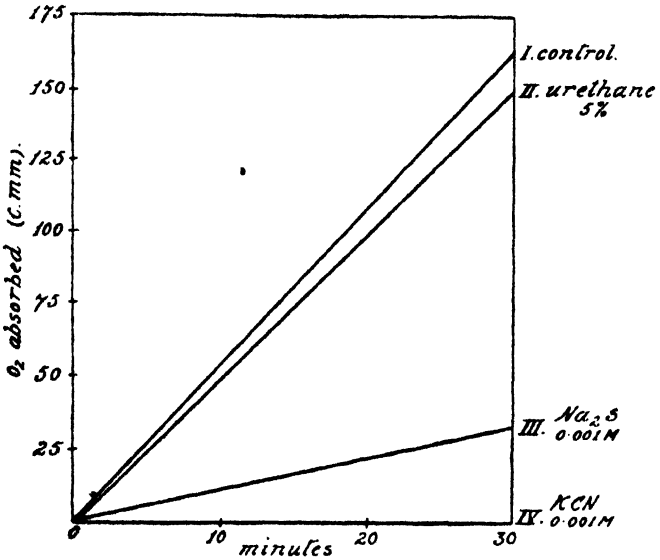

FIG. 6.—Oxygen uptake by oxidase + cytochrome + cysteine: I, control; II, in presence of 5 per cent. of ethyl urethane; III, in presence of 0·001 M. of Na_2S; IV, in presence of 0·001 M. of KCN. (See experiment 1, Table IV.)

Table IV.

Experiment No.	t.	Time.	Muscle oxidase.	Cytochrome.	Cysteine.	Inhibitors.	O_2 uptake	Percentage of inhibition.
	° C.	mins.	c.c.	c.c.	mg.		c.mm.	
1	21	30	1·5	0·2	8	0	161	0
			1·5	0·2	8	0·57 M. urethane	150	7
			1·5	0·2	8	0·001 M. Na_2S	33	80
			1·5	0·2	8	0·001 M. KCN	0	100
2	18	33	1·3	0·2	8·5	0	182	0
			1·3	0·2	8·5	0·57 M. urethane	172	5·5
3	16	40	1·5	0·2	7	0	101	0
			1·5	0·2	7	0·001 M. Na_2S	13	87

the same concentration of Na_2S inhibits the reaction by 80 to 87 per cent. The incomplete inhibition by Na_2S may be due to the presence in the system of some metals, or even autoxidisable hæmatin compounds the catalytic activity of which towards cysteine, as was shown recently by Krebs (1929) instead of being inhibited by Na_2S, is on the contrary increased. Narcotics such as ethyl urethane, on the other hand, have a very small effect on this

system, and the slight inhibition by about 5 to 8 per cent. of the rate of oxygen uptake produced by 5 per cent. of ethyl urethane was mainly due to the partial aggregation of the muscle particles of the oxidase preparation.

VII. *Effect of Carbon Monoxide.*

It was shown by Warburg (1926) that CO in high concentration inhibits considerably the oxygen uptake of living cells and that this inhibition is diminished with the increase of the partial pressure of O_2 or when the cells are exposed to light. It was also shown (Keilin, 1927, 1929) that carbon monoxide has the same inhibitory effect on the indophenol oxidase of the yeast and of the heart-muscle cells. In view of these results it was important to test the effect of CO on the catalytic oxidation of cysteine by the reconstructed oxidase-cytochrome system.

The experiments on the effect of CO on the oxidase-cytochrome system were carried out in four differential manometers, the left-hand flasks of which received 3 c.c. of buffer solution. The right-hand flask of the first manometer received oxidase preparation and cysteine; of the second, cytochrome and cysteine; of the third and the fourth oxidase, cytochrome and cysteine. By a method described in a previous paper (1929, p. 214) the first three manometers were filled with a gas mixture composed of $8\ N_2 + 1\ O_2$, the fourth manometer was filled with a mixture of $8\ CO + 1\ O_2$. The oxygen uptake, measured in dark at 17° C. in 40 minutes, was as follows:—

In gas mixture $\frac{N_2}{O_2} = 8$:—

(*a*) Oxidase + cysteine took up .... 21·5 c.mm.
(*b*) Cytochrome + cysteine took up .... 14·0 „
(*c*) Oxidase + cytochrome + cysteine took up.. 165·0 „

In gas mixture $\frac{CO}{O_2} = 8$:—

(*d*) Oxidase + cytochrome + cysteine took up.. 107·0 „

In a preliminary experiment it was found that CO has no effect upon the oxygen uptake of cysteine in the presence of either oxidase or cytochrome alone. To estimate the effect of CO on the oxidase-cytochrome system we have to subtract from the oxygen uptake given by this system the oxygen uptake

given separately by oxidase-cysteine and cytochrome-cysteine, which will give :—

$$\text{in } \frac{N_2}{O_2} = 8\,;\ A_0 = c - (a + b) = 129\cdot5 \text{ c.mm.}$$

$$\text{in } \frac{CO}{O_2} = 8\,;\ A = d - (a + b) = 71\cdot5 \text{ c.mm.}$$

The inhibition produced by CO in this mixture is by about 45 per cent. (fig. 7) and the partition constant obtained from the equation introduced by Warburg (1927) is as follows :—

$$K = \frac{n}{1-n}\cdot\frac{CO}{O_2}\,;\quad n = \frac{A}{A_0}\quad K = \frac{71\cdot5}{129\cdot5 - 71\cdot5} \times 8 = 9\cdot9.$$

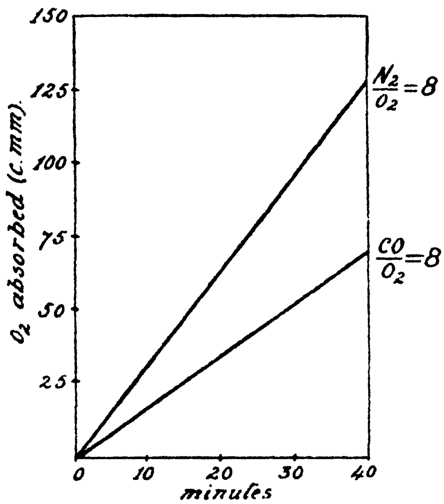

FIG. 7.—Effect of carbon monoxide on oxidation of cysteine by oxidase-cytochrome system : I, oxygen uptake in gas mixture, $8\,N_2 + 1\,O_2$; II. oxygen uptake in gas mixture of $8\,CO + 1\,O_2$.

In a similar way it was found that the oxygen uptake of cysteine in 40 minutes in the presence of the oxidase-cytochrome system in air was 166 c.mm., while in a gas mixture composed of $4\,CO + 1\,O_2$ it was 123 c.mm. The inhibition due to CO was 27 per cent. and the partition constant $K = 10\cdot8$.

To study the effect of light on CO inhibition the differential manometers, with the flasks filled in the same manner as in the previous experiments, were shaken in a bath, with a glass wall and a submerged mirror receiving the light from a 100-c.p. half-watt lamp standing outside the bath and projecting it on to the flasks of the manometers. By means of movable screens, in a few

seconds the bath could be either completely darkened or exposed to strong light. The oxygen uptake was measured for 40 minutes with the flasks shaken in dark, followed by 20 minutes with the flasks exposed to light and 30 minutes again in dark. The results of this experiment are shown in Table V and fig. 8.

Table V.

Time.	Gas mixture.	O_2 uptake.	Percentage of inhibition.	$K = \frac{n}{1-n} \cdot \frac{CO}{O_2}$.
minutes		c. mm.		
40 Dark	$\frac{N_2}{O_2} = 8$	120	0	—
	$\frac{CO}{O_2} = 8$	61	49	8·3
20 Light	$\frac{N_2}{O_2} = 8$	62	0	—
	$\frac{CO}{O_2} = 8$	55	11	63
30 Dark	$\frac{N_2}{O_2} = 8$	90	0	—
	$\frac{CO}{O_2} = 8$	45	50	8

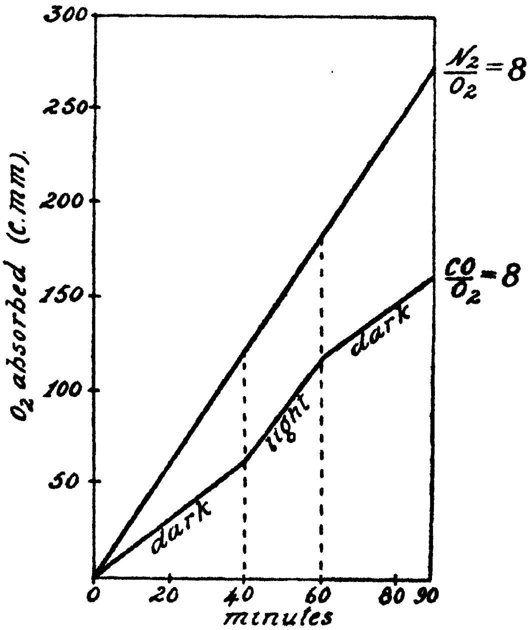

FIG. 8.—Effect of carbon monoxide in dark and in light on the oxidation of cysteine by oxidase-cytochrome system. (See Table V.)

From the above experiments it follows that the oxidation of cysteine in dark by the oxidase-cytochrome system is greatly inhibited by carbon monoxide and that the inhibition is much reduced when this system is exposed to light. In other words, the reconstructed oxidase-cytochrome system in the presence of a high concentration of CO behaves in a very similar way to the oxidase-cytochrome of intact living cells, or to the total respiration of intact yeast cells, as was shown by Warburg (1926, 1927).

VIII. *Protein Fraction of the Cytochrome Preparation.*

Cytochrome preparation, as was previously shown (p. 422), in addition to the pigment contains variable amounts of proteins. It was, therefore, important to find whether some other constituent of these preparations could form with oxidase a catalytically active system. For this purpose, by a method previously described, two separate fractions of precipitate were obtained from the cytochrome preparation of stock A. The first fraction, which we shall call P, formed a heavy, flocculent, white precipitate, easily soluble in weak alkali, giving a pale yellow solution with hardly perceptible bands of cytochrome *c*. The second fraction, which we shall call C, formed a brown precipitate, giving, when neutralised, a deep red solution with a very strong absorption spectrum of cytochrome *c*. Two separate solutions of the precipitates P and C have been prepared, solution P made if anything much stronger in protein than the solution C. The experiment was carried out in the usual way in four Barcroft manometers, the right-sided flasks of which received: in the first manometer, 0·1 c.c. of cytochrome fraction C and 8·5 mg. of cysteine; in the second manometer, 0·1 c.c. of the same cytochrome solution, 8·5 mg. of cysteine and 1·5 c.c. of the usual muscle oxidase preparation; in the third manometer, 0·1 c.c. of the protein solution P and 8·5 mg. cysteine; and in the fourth manometer, 0·1 c.c. of solution P, 8·5 mg. of cysteine and 1·5 c.c. of oxidase preparation. The oxygen uptake in 40 minutes at 17° C. (fig. 9) was as follows:—

Cytochrome (fraction C) + cysteine	took up	5·6	c.mm. of O_2
Cytochrome (fraction C) + oxidase + cysteine..	,,	134·0	,,
Protein (fraction P) + cysteine	,,	10·6	,,
Protein (fraction P) + oxidase + cysteine	,,	18·7	,,

In another experiment the oxygen uptake was measured in two differential manometers, the left flasks of which received 8 mg. of cysteine with 0·2 c.c. of cytochrome (C) or protein (P) solutions; while the right flasks, in addition

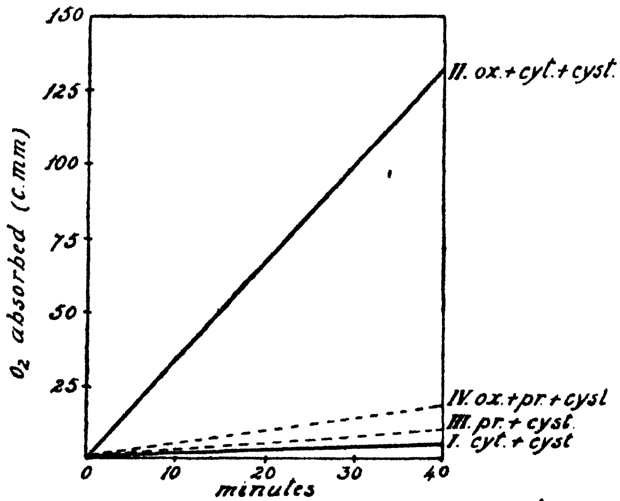

Fig. 9.—Oxygen uptake by cysteine in presence of: I, cytochrome alone; II, oxidase and cytochrome; III, protein fraction from cytochrome preparation; IV, oxidase and protein fraction.

to these reagents, received 1·5 c.c. of muscle oxidase preparation. In 25 minutes at 17° C. cytochrome (fraction C) + cysteine + oxidase took up 128 c.mm. of O_2; while protein (fraction P) + cysteine + oxidase took up 15 c.mm. of O_2.

These experiments clearly demonstrate that the activity of cytochrome preparation does not depend on the bulk of precipitate obtained from stock A, but only on the actual concentration of the pigment.

IX. *Effect of Sodium Pyrophosphate.*

It was mentioned previously that muscle preparation contains more catalytically active metals than it shows by the direct oxidation of cysteine. Muscle preparation seems to contain substances which are capable of combining with these metals in such a way as to remove them from direct activity, and this property of the muscle preparation has made the experiments with the oxidase-cytochrome system easy to carry out and to interpret. It is possible, however, to bring these metals into action simply by the addition to the muscle preparation of a small amount of sodium pyrophosphate. This can be easily demonstrated by a series of experiments similar to those previously described, in which cytochrome *c* is replaced by sodium pyrophospate.

A typical experiment is carried out in four differential Barcroft manometers. The left flasks of the manometers received 3 c.c. of phosphate buffer (pH 7·8);

the right flask of the first manometer received 8 mg. of cysteine; of the second, 8 mg. of cysteine and 1·5 c.c. of muscle oxidase preparation; of the third, 8 mg. of cysteine, 1·5 c.c. of oxidase and 0·02 M. sodium pyrophosphate; and of the fourth, 8 mg. of cysteine and 0·02 M. pyrophosphate. All the flasks were made up to 3 c.c. with phosphate buffer, and 0·3 c.c. of 5 per cent. KOH was added as usual to the tubes sealed at the bottoms of the flasks. In this experiment the oxygen uptake of cysteine in 40 minutes at 21° C. was as follows:—

(1) Cysteine alone	9·6	c.mm. of O_2
(2) Cysteine + oxidase	52·0	,,
(3) Cysteine + oxidase + pyrophosphate	193·0	,,
(4) Cysteine + pyrophosphate	7·4	,,

The experiment shows clearly that the rate of oxidation of cysteine in muscle preparation is greatly increased by the addition of sodium pyrophosphate. The effect produced by this salt does not vary much with its concentration if used within the limits of 0·1 M. and 0·003 M., but the effect rapidly falls off below this concentration.

The oxidation of cysteine in the presence of muscle preparation and pyrophosphate is inhibited on the addition of 0·001 M. of KCN by at least 92 per cent.; Na_2S, on the other hand, inhibits it only by 58 per cent. Warming or even boiling muscle preparation with pyrophosphate has very little effect on this system. For instance, while a fresh sample of muscle preparation with pyrophosphate and cysteine took up 156 c.mm. of O_2, a similar sample of muscle and pyrophosphate heated to 90° C., or even boiled before the experiment, took up 145 c.mm. of O_2. The catalytic activity of this system is not affected by carbon monoxide, even if kept in dark.

All this shows that we are dealing here not with the oxidase system but with a metal which is present in an inactive state in the muscle preparation and which is brought into activity by the addition of pyrophosphate. That the metal responsible for this catalytic oxidation is copper can be clearly understood in the light of the work of Warburg (1927), who has shown that while pyrophosphate inhibits the oxidation of cysteine by iron it has no effect on the oxidation of cysteine by copper. Moreover, metal-free pyrophosphate and cysteine have been used with success by Warburg for the detection of the minute quantities of copper in organic fluids; he also noticed that these fluids are capable of depressing the catalytic activity of copper alone.

Pyrophosphate, as is known, forms a compound with copper and this

compound seems to have the same catalytic properties towards cysteine as other salts of copper.

As the muscle preparation contains copper in addition to the oxidase it was interesting to find how this preparation would behave when cytochrome and pyrophosphate were added simultaneously. One of these experiments, carried out in three differential manometers, gave the following results :—

1·5 c.c. of oxidase + 8 mg. cysteine + 0·2 c.c. cytochrome *c* in 16 minutes took up	106 c.mm. O_2
1·5 c.c. oxidase + 8 mg. cysteine in 0·1 M. sodium pyrophosphate in 16 minutes took up....................	76 ,,
1·5 c.c. oxidase + 8 mg. cysteine + 0·2 c.c. cytochrome *c* + 0·1 M. sodium pyrophosphate in 16 minutes took up	217 ,,

This experiment shows that the rate of oxygen uptake of cysteine in the presence of muscle oxidase preparation, pyrophosphate and cytochrome *c* is even slightly greater than the sum of the rates of oxygen uptake of the two systems : (1) muscle preparation with cytochrome ; and (2) muscle preparation with pyrophosphate measured separately.

As the pyrophosphate was already present in the concentration of its maximum efficiency, the greatly increased oxygen uptake on addition of cytochrome shows that the oxidase with cytochrome forms a completely independent catalytic system.

X. *Discussion and General Conclusions.*

The experiments discussed in this paper show that the oxidase itself does not react directly with substances undergoing oxidation. Unlike all known autoxidisable hæmatin compounds, the oxidase does not catalyse directly the oxidation of cysteine. The latter is, however, rapidly oxidised by the oxidase in the presence of cytochrome, and, what is more important, in the presence of its non-autoxidisable component *c*.

The direct observation of the living cells, as well as the experiments *in vitro*, show that oxidase reacts specifically with the intracellular hæmatin compounds,* forming with them an active catalytic system.

* Oxidase of muscle preparation reacts also with helicorubin, a hæmatin compound found in the liver and gut of snails and other invertebrates. Reduced helicorubin, which has a characteristic absorption spectrum of a hæmochromogen compound, does not combine with CO. It easily oxidises in slightly acid solution, but not in alkaline solution, even if shaken vigorously with air. When, however, the alkaline solution of the reduced helicorubin is brought in contact with muscle suspension, like cytochrome, it immediately becomes oxidised.

It oxidises also another type of hæmochromogen compound, which, instead of cytochrome, is found in facultative anaërobes, such as *Bacillus coli*, in liver cells of mammals, and in the cells of some invertebrates (Keilin, 1929).

The results of this study confirm also our previous observations, namely, that the properties and the activity of the oxidase in heart-muscle preparation can be studied independently from those of other respiratory enzymes of the cell such as dehydrogenases. The oxidase-cytochrome system in our preparation has the same properties as it has in the living cells, and this can be confirmed step by step, using cytochrome, a visible cell constituent, as an indicator of the behaviour of this system.

All the factors which affect the oxidation of cytochrome in the living cell affect in the same way and to the same degree the respiration of cells and the oxidation of cysteine by the oxidase-cytochrome system reconstructed from the oxidase preparation of heart-muscle and cytochrome *c* of bakers' yeast. Like the respiratory activity of the cells, the activity of this oxidase-cytochrome system towards cysteine is completely abolished by heating the enzyme above 70° C. and is greatly inhibited by very small concentrations of KCN and of H_2S.* Like respiration, it is inhibited in dark by a high concentration of CO, but is reactivated when exposed to light or to a higher partial pressure of oxygen. The partition constant of this system between O_2 and CO is of the same magnitude as that which was obtained by Warburg in his study of intact yeast cells.

Narcotics, however, have little or no effect on the activity of the oxidase-cytochrome system. This confirms our previous observations (1925, 1929), namely, that narcotics, while they inhibit the respiration of intact cells do not affect the oxidation of reduced cytochrome; they inhibit, however, the activity of dehydrogenases and therefore the reduction of oxidised cytochrome.

The oxidase, which is inhibited by KCN and not by narcotics, and the dehydrogenases, which are inhibited by narcotics but not by KCN, etc., form two portions of the same respiratory system of the cell, having as a function the oxidation of organic metabolites by molecular oxygen. The term respiration

* In this relation it may be noted that according to Krebs (1929) the oxidation of cysteine by hæmatin compounds is inhibited by KCN and accelerated by H_2S, while the oxidation of linoleic acid is inhibited by H_2S. On the other hand, the oxidation of linseed oil (Robinson, 1924) and of olive oil (Kuhn and Meyer, 1929) by hæmatin is not inhibited by KCN. In other words, the effects of KCN and H_2S on catalytic oxidation produced by autoxidisable hæmatin compounds depend on the substrates undergoing oxidation (Kuhn and Meyer).

covers the activity of the whole system and therefore cannot be applied to only one portion of this system.

The fact that the oxidase-cytochrome system of our preparation does not catalyse the oxidation of all the metabolites which are so easily oxidised in the living cell does not mean that the oxidase was decomposed, damaged or changed in any way. It means only that in our preparation it was separated from other activating mechanisms of the cell, which, together with the oxidase-cytochrome system, are essential for the oxidation of these metabolites.

On treating the cells or tissues in various ways one can desaturate, disorganise, or even destroy, various activating mechanisms, leaving the oxidase-cytochrome system undamaged. In this case the oxidase-cytochrome system will oxidise only substances such as cysteine, paraphenylenediamine, catechol, etc., which do not require activation by dehydrogenases.

The catalytic activity of the oxidase-cytochrome system—estimated in terms of cubic millimetres of oxygen transferred per gramme of muscle preparation per hour at 20° C. to cysteine in the presence of cytochrome *c*—varies from 5000 to 7500 c.mm. or more. The activity of the oxidase-cytochrome system falls, therefore, within the range of the oxygen uptake of intact heart, which, according to Evans and Matsuoka ('Journ. of Physiol.,' vol. 49, 1915), takes up at 37° C. 3240 c.mm. of oxygen per gramme of heart per hour in an animal at rest and 3 times more during exercise.

The attempt can be made to estimate the catalytic activity of oxidase-cytochrome towards cysteine in terms of Fe of cytochrome. This can be done only approximately, as the concentration of cytochrome in our experiments was only roughly estimated by comparing its colour and absorption spectrum with a series of known solutions of pyridine mesohæmochromogen. Estimated in this way it was found that 0·015 mg. of cytochrome *c*, corresponding to approximately 0·0012 mg. of Fe can transfer in air in 60 minutes at 18° C. to only 8 mg. of cysteine, 241 c.mm. of oxygen. The activity of this system represented in terms of cubic millimetres of O_2 transferred to cysteine per milligramme of iron of cytochrome per hour at 18° C. will be approximately 200,000, which is of the same magnitude as the activity of nicotine hæmochromogen towards ferro-cysteine, as was found by Krebs (1929).

All this gives, however, a very incomplete indication as to the catalytic efficiency of oxidase-cytochrome system within the living cell. In intact cells, with the three components of cytochrome and unbound hæmatin, cysteine is not a normal metabolite for oxidation, there this system reacts mainly and very rapidly with organic molecules activated by dehydrogenases. Naturally,

one of the important factors which come into play when we consider the cellular respiration is the proper distribution of all the components of this system, and this is one of the properties of cellular structure. In the living cell with its normal distribution of activating mechanisms and normal metabolites a much smaller concentration of cytochrome will be required for the same velocity of oxidation than that used in our experiments, where oxidase partly exposed on the surfaces of muscle particles, cytochrome *c* extracted from yeast cells, and an artificial substrate such as cysteine had a more or less haphazard distribution.

XI. *Summary.*

(1) Component *c* of cytochrome can be extracted from bakers' yeast cells, giving a strong transparent solution of a deep red colour.

(2) In this extract cytochrome *c* is not autoxidisable and has the same absorption spectrum and the same properties as it shows in intact living cells.

(3) Cytochrome *c* thus extracted, when made free from metallic impurities, does not catalyse the oxidation of cysteine.

(4) Oxidase of heart muscle preparation, unlike autoxidisable hæmatin compounds, does not oxidise cysteine.

(5) Oxidase of heart muscle and cytochrome *c* of yeast cells when brought together form a powerful catalytic system which rapidly oxidises cysteine.

(6) The activity of this system, like the respiratory activity of the cell, is abolished by warming above 70° C., it is almost completely inhibited by 0·001 M. of KCN, and it is inhibited by 80–87 per cent. in the presence of 0·001 M. of Na_2S.

(7) It is also inhibited in dark by high concentrations of carbon monoxide, the partition constant K between this system, oxygen and carbon monoxide varying between 8 and 10, which is of the same magnitude as that obtained by Warburg from the study of the respiration of intact cells.

(8) Strong light diminishes the inhibitory effect produced by CO, shifting the partition constant K from 8 to 63.

(9) Urethane, however, has no effect on this system ; and this confirms our previous results, namely, that the effect of narcotics on respiration is mainly due to their inhibition of the activity of dehydrogenase system of the cell.

(10) The catalytic system reconstructed from the oxidase of heart muscle and cytochrome *c* of yeast cells behaves, therefore, like a true respiratory system of the cell. This is, moreover, supported by observations discussed in previous papers, namely :—

(*a*) That cytochrome and other spectroscopically detectable hæmatin compounds are very widely if not universally distributed in cells of aerobic organisms.

(*b*) That the oxidation and reduction of cytochrome can be directly observed in intact living cells (bacteria, yeast, insects).

(*c*) Finally, that under biological conditions cytochrome reacts on the one hand with intracellular oxidase, on the other with organic molecules or metabolites activated within the cells by dehydrogenases.

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The Vapour Pressure of Muscle.

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I. Introduction.

In October, 1927, one of us (A.V.H.) noticed a curious phenomenon connected with the rate of resting heat production of a muscle suspended in pure nitrogen. The muscle, previously soaked for an hour or more in Ringer's solution on a thermopile, in order (*a*) to quicken temperature equalisation in the chamber, and (*b*) to ensure its good condition,† showed a heat-rate in nitrogen, on removing the solution, which he believed, and which we still believe, to be a true measure of the resting anaerobic metabolism. When stimulated the muscle liberated heat; on the cessation of the stimulus this heat was dissipated and the galvanometer recording the temperature of the muscle returned towards, but not to, its original position. In hundreds of experiments, with no exception, the resting heat-rate in nitrogen after stimulation was greater than before. The phenomenon was obvious: it showed exact quantitative relations and was discussed in various papers (Hill, 1928*b*, 1928*c*, 1929*a*, 1929*b*; Hartree and Hill, 1928). It seemed to be a sign of disintegrative chemical reactions, resulting from anaerobic activity. If oxygen was admitted, after the completion of the usual recovery process the heat-rate in nitrogen, later reintroduced, returned towards, or to, its original level. This appeared to show that oxygen creates, or maintains, a barrier of some kind to disintegrative reactions which proceed in its absence.

At an early stage the results were communicated to Prof. Meyerhof, then in Berlin, who attempted to confirm them by another technique, viz., by comparing the resting heat production of muscles in a closed calorimeter, before and after stimulation. The attempt was entirely unsuccessful—there was no sign of an increment in heat-rate, after anaerobic stimulation, comparable with that invariably observed with the thermopile. His results (Meyerhof,

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† Actually, as it proved, in order to avoid the reversible inexcitability described later by Dulière and Horton (1929).

McCullagh and Schulz, 1930) have been published recently. This failure obviously demanded the most critical examination of the thermoelectric technique; no flaw, however, was found in it, and the phenomenon occurred unchanged when a sensitive resistance thermometer was substituted for the thermopile. In the meantime other characteristic properties of it were observed; the increment in heat-rate due to anaerobic stimulation had a temperature coefficient per 10° C. of about 2; it could be abolished not only by recovery in oxygen, but by prolonged soaking in Ringer's solution (even oxygen-free); and finally in hydrogen* it was 3 to $3\frac{1}{2}$ times as great as in nitrogen. This last astonishing effect supplied the clue to the explanation, which was given in a postscript to a paper (Hill, 1929*b*) published in September, 1929. The phenomenon is due entirely to a change of vapour pressure of the muscle, caused by the increase in the number of dissolved molecules (or ions) resulting from anaerobic stimulation.

The importance in myothermic experiments of the vapour pressure of the muscle had not previously been realised. Not till the adoption of the instruments and methods now in use had freedom from galvanic and other disturbances been sufficient to allow an accurate measurement to be made of the absolute value of the resting heat-rate. It had been recognised empirically that it is advisable to give the muscle a long preliminary soaking in Ringer's solution in the thermopile chamber; without such a soaking it may be many hours before even an approximate constancy of the resting heat-rate is reached. This soaking, it is now clear, brings the muscle into vapour pressure equilibrium with the Ringer's solution; without it, an exact equilibrium is attained only very slowly by the transfer of water as vapour through the gas in the chamber. For a long time our colleague Mr. Hartree, working at Cambridge with the older type of vulcanite thermopile chambers, has observed that, even after prolonged soaking, the muscle suspended in oxygen or nitrogen shows a continual temperature drift in the negative direction. This is now seen to be due to a gradual concentration of the salt in the droplets of solution on the walls of the chamber, owing to absorption of water by vulcanite, leading to a fall of vapour pressure; the surface of the muscle, therefore, is subjected to slow evaporation, increasing as time goes on. The amount of this evaporation is not

* Hydrogen had been avoided previously, lest by diffusing into the hollow space inside the thermopile faster than the air could diffuse out, it might blow the thermopile out and destroy it. This actually happened when hydrogen was finally tried with the thermopile; the observations with hydrogen were made with the resistance thermometer, which was not hollow.

large enough to have any effect on the muscle itself during an experiment; it is large enough, however, to cause an appreciable and increasing negative heat-rate with the delicate instruments employed. Mr. Hartree has found it to be abolished by coating the surface of the vulcanite with a film of paraffin wax.

This effect of vapour pressure is not small. If a suitable symmetrical ("differential") thermopile with 70 couples, similar in general type to that described by Downing and Hill (1929) for the measurement of nerve heat, have one set of junctions covered with a piece of filter paper soaked in 1 p.c. NaCl and the opposite set with a similar piece of filter paper soaked in distilled water, and if it be then mounted in a moist chamber at 20° C. and connected to a galvanometer of sensitivity 1 mm. $= 1 \cdot 5 \times 10^{-10}$ amp., within 30 to 45 minutes it will show a steady deflection of 1200 mm. on the scale. The difference between the vapour pressures of the two fluids determines a difference between their rates of evaporation and consequently a difference between the temperatures of the two faces of the thermopile. The phenomenon has been made the basis of a sensitive and rapid method of finding molecular weights, or molal concentrations, in small quantities of fluid (Hill, 1930). This method has been employed for the investigation of the "free" water of muscles and of various protein solutions as described in the following paper by Hill.

To illustrate the sensitivity of the myothermic apparatus to changes of vapour pressure, if the solution on the walls of a chamber containing a muscle on an ordinary thermopile be altered from Ringer's solution of normal concentration to one of 60 p.c. normal concentration, the apparent resting heat-rate increases by 200 mm. or more on the scale of the galvanometer referred to above; the heat-rate due to the true anaerobic resting metabolism is only 20 to 40 mm. on the same scale.

The effect of a dissolved substance on the vapour pressure is best expressed in terms of the "relative molal depression" $(p_0 - p)/mp_0$, where p_0 is the vapour pressure of the pure solvent, p that of the solution, and m the molal concentration of the solute (gramme molecules per 1000 grammes of solvent). According to data in the International Critical Tables, vol. III, the relative molal depression of vapour pressure of a 0·7 p.c. solution of NaCl is about 0·0330, so that the relative depression $(p_0 - p)/p_0$ is 0·00395. This may seem very small; at 20° C. it amounts to only 0·069 mm. of Hg pressure. Nevertheless a muscle isotonic with 0·7 p.c. NaCl in a chamber moist with 0·42 p.c. NaCl (the case referred to above) may have an apparent resting heat-rate some 200 mm. too great, as the result of condensation of water vapour upon it. The difference between the vapour pressures of 0·7 p.c. and 0·42 p.c. NaCl

solutions at 20° C. is 0·028 mm. of Hg, so that 1 mm. on the galvanometer scale corresponds to a vapour pressure difference of $1 \cdot 4 \times 10^{-4}$ mm. $= 0 \cdot 14\ \mu$ of Hg.

The absolute smallness of the change of vapour pressure involved is the only excuse which can be offered for the failure by one of us to recognise its importance before. Nobody had realised the extreme sensitivity of the myothermic method in this respect, or the fact that the condensation of even 1 mg. of water in 24 hours on a muscle of 150 mg. would lead to an extra rate of heat production of 118 g.cm. per g. per minute, which is about twice the true rate due to its anaerobic metabolism at 20° C., and might cause an extra deflection of 50 mm. on the galvanometer scale. An extreme value for the increment in resting heat-rate in nitrogen, due to an exhausting stimulus, is 1000 g.cm. per g. per minute. This represents the condensation of only about 9 mg. of water *per day* on a muscle of 150 mg., a rate which in the few hours of an experiment it would be very difficult to measure.

II. The absolute value of the Osmotic Pressure of Frogs' Muscles and Blood.

The osmotic pressure P of a solution is related to its vapour pressure (for not too great concentrations) by the equation

$$\log_e p_0/p = \mathrm{PV}/\mathrm{RT},$$

where p_0 is the vapour pressure of the pure solvent, p that of the solution, P the osmotic pressure and V the volume occupied in the liquid state* by one gramme molecule of the vapour of the solvent. For the case of dilute solutions in water this equation may, with sufficient accuracy for most purposes, be written

$$[(p_0 - p)/p_0]_{\mathrm{T}} = \mathrm{P}/4 \cdot 56\mathrm{T} \qquad (1)$$

where P is reckoned in atmospheres, and $[(p_0 - p)/p_0]_{\mathrm{T}}$ is the relative lowering of vapour pressure at absolute temperature T.

We see that the osmotic pressure may be simply calculated from the relative depression of vapour pressure $(p_0 - p)/p_0$. We shall not need, therefore, in what follows, to refer directly to osmotic pressures as such ; it is more convenient to deal with depressions of vapour pressure. When referring, however, to the solute, and its effect in causing a depression of the vapour pressure of

* To be precise, V is the increase of volume caused by condensing in a large quantity of the solution 1 g. mol. of the vapour of the solvent.

the solvent, we shall often retain the convenient term "osmotic pressure." Usually, moreover, it will be unnecessary to refer directly to the depression of vapour pressure. The determining factor in a physiological salt solution is the concentration of NaCl, and the relative depression of vapour pressure of a solution of NaCl is simply calculated from its molal concentration, by means of the following values of the relative molal depression interpolated from the International Critical Tables, vol. III :—

Per cent. NaCl	0·4	0·5	0·6	0·7	0·8
$(p_0 - p)/mp_0$	0·0333	0·0332	0·0331	0·0330	0·0329
Per cent. NaCl	0·9	1·0	1·1	1·2	1·3
$(p_0 - p)/mp_0$	0·0329	0·0329	0·0328	0·0328	0·0328

We shall deal, therefore, with osmotic and vapour pressures mainly by referring to the corresponding solutions of NaCl.

It will be noticed that usually we employ *molal* concentrations—g. mols. of solute per 1000 g. of solvent—or express the strengths of our solutions in grammes of solute per 100 g. of solvent. In dealing with problems of vapour pressure this is customary; simpler relations are found between quantities expressed in this way. This is particularly the case when dealing with solutions such as blood and muscle, which contain a large proportion of solid material.

Attempts have been made by many observers to determine the exact strength of a solution of NaCl which is isotonic with frogs' muscles. Cooke (1898), by measuring the changes in weight of a frog's resting gastrocnemius suspended in various NaCl solutions, found it to be isotonic with 0·8 p.c. NaCl, the fatigued muscle to be isotonic with 1·1 p.c. NaCl. Overton (1902), however, by similar methods found the frog's resting sartorius to be isotonic with 0·7 p.c. NaCl and disputed the accuracy of Cooke's results. Fletcher (1904) obtained a value of 0·75 p.c. and, like Cooke, found the fatigued muscle to swell far more rapidly than the resting muscle when placed in a hypotonic solution; he also noted that the curve of swelling as a function of time is different.

The depression of freezing point of frogs' muscles has been determined by various workers. Jensen and Fischer (1910), employing chiefly *R. esc.*, gave ΔT as lying between 0·46° and 0·53°. Jensen (1912, p. 349) found the expressed juice of muscle to freeze at —0·40° C., and gave (p. 353) —0·45° C. as the usual freezing point of muscle. Brunow (1912), employing *R. fusca*

regularly observed a ΔT of $0 \cdot 42°$. Moore (1916) also found a mean value of $0 \cdot 42°$ for the resting muscles of the American bull frog. Taking $\Delta T = 0 \cdot 43°$ as the most probable value, and assuming for NaCl in this region of concentration a molal depression of freezing point of $3 \cdot 46°$ (Landolt-Börnstein), we may calculate that resting muscle freshly removed from the body is isotonic with a sodium chloride solution containing $0 \cdot 725$ g. of NaCl in 100 g. of water.

There are possible objections to both methods of determining the strength of a solution of NaCl which has the same osmotic pressure as muscle: (*a*) the measurement of the change of weight of a muscle immersed in a given solution requires considerable periods of immersion and is not very accurate; secondary effects moreover may arise, at any rate in the later stages of soaking; the only really safe fluid to immerse a muscle in is the animal's own blood; (*b*) the freezing point method, strictly speaking, only measures the osmotic pressure at the freezing point; to apply the results to other temperatures involves the assumption that the physical state of the solvent and of the various solutes in the living cell is unaffected by a change of temperature. It is desirable, therefore, to employ some independent method to verify the results obtained by these two.

A resting muscle is necessarily in osmotic equilibrium with the blood which is circulating through it. The depression of vapour pressure of blood can be determined at any desired temperature by the method recently described (Hill, 1930) and referred to above, and in more detail in the succeeding paper by Hill. This depression can be compared with that of a given solution of NaCl. In practice it is more convenient and accurate to read directly the small *difference* of vapour pressure between the sample of blood in question and a solution known to be approximately isotonic with it.

The solution used for comparison contained $0 \cdot 708$ g. of NaCl in 100 g. of water. The frogs employed were *R. esc.*, either of the large Hungarian or the smaller Dutch variety. Blood was collected in a syringe by destroying the animal's brain with a seeker, exposing the heart and inserting a hypodermic needle into one aorta. Muscular activity increases the osmotic pressure of the muscles and of the blood circulating through them; every precaution therefore was taken to avoid struggling of the animal before or after decerebration, and to reduce the time of collecting the blood to a minimum. An anæsthetic could not be used for fear of changing the osmotic pressure. Two thermopiles were employed in every case, and a mean of the readings taken. On one face was placed a piece of filter paper dipped in the sample of blood, on the opposite face a similar piece dipped in the NaCl solution. The difference

of vapour pressure, positive or negative, was interpreted in terms of the strength of a NaCl solution, by calibrating the thermopiles with a known solution on one face against water on the other. The results are given in Table I.

Table I.

The osmotic pressure of frogs' blood, expressed in terms of the solution of NaCl (g. of NaCl in 100 g. H_2O) which has the same vapour pressure at 20·25° C. H = Hungarian *R. esc.*; D = Dutch *R. esc.* All experiments in January, 1930.

Number	1	2	3	4	5	6	7
Frog	D	D	H	H	D	D	D
Strength of isotonic solution	0·772	0·762	0·721	0·723	0·867	0·733	0·784

Number	8	9	10	11	12	13
Frog	D	D	D	D	H	H
Strength of isotonic solution	0·715	0·739	0·783	0·705	0·686	0·688

The abnormal result in experiment No. 5 was obtained with both instruments, presumably, therefore, it was not due to experimental error, but to some peculiarity of the blood. Omitting it, and experiment No. 1 in which there was appreciable struggling before the blood was taken, the mean value is 0·731. Any error, *e.g.*, struggling of the animal or exposure of the blood, would tend to raise the osmotic pressure, so we may regard the mean isotonic solution as being one containing not more than 0·731 g. of NaCl in 100 g. of H_2O, or expressed in the usual way 0·726 p.c. NaCl. This is very nearly the same as the value calculated above from the freezing point.

In experimental work with isolated muscles, in order to avoid changes of *p*H due to bubbling gas through the solution, it is common to work with CO_2- and bicarbonate-free Ringer's fluid, buffered if necessary with phosphate. The total CO_2 content of resting frog's muscle freshly taken from the body is about 12 volumes p.c. This CO_2 is removed by soaking in the Ringer's fluid referred to, and consequently the total number of ions and molecules in solution in the muscle is reduced. Now 12 c.c. of CO_2 dissolved in 77 c.c. of water (the "free" water contained in 100 g. of muscle—see the following paper by Hill) exerts about the same osmotic pressure as 0·022 p.c. of NaCl. Thus a bicarbonate-free Ringer's fluid which is to be isotonic with muscle after removal of the CO_2 contained in the latter should have the same osmotic pressure, not as

0·726 p.c., but as 0·704 p.c. NaCl. The Ringer's solution used in the later part of the present investigation was as follows :—

NaCl	0·675 p.c.
$CaCl_2$	0·020 p.c.
KCl	0·015 p.c.

This can be calculated to have the same osmotic pressure as 0·703 p.c. NaCl. In the earlier experiments, however, before the observations and calculations given above were made, the following Ringer's fluid was used :—

NaCl	0·650 p.c.
$CaCl_2$	0·0125 p.c.
KCl	0·014 p.c.

The osmotic pressure of this is definitely too low, being equal to that of 0·671 p.c. NaCl.

[*Added in proof.*—The results of Table I are probably about 0·01 to 0·02 too low. The blood should have been exposed to air containing about 2½ p.c. of CO_2. Recent experiments by Margaria (unpublished) on mammalian blood have shown that a measurable fall of osmotic pressure is caused by removing the combined CO_2 by exposing the blood to CO_2-free air.]

III. The Soluble Constituents of Muscle, Resting and Fatigued.

The concentrations of the cations present in frogs' muscles, and of chloride and water, according to Meigs and Ryan (1912) (*R. catesbiana*), and according to Katz (1896) (*R. esculenta*) are as follows, expressed in mg. per 100 g. of fresh muscle :—

	K.	Na.	Ca.	Mg.	Cl.	H_2O.
Meigs and Ryan	350	54	28	30	66	79900
Katz	308	55	16	23	40	81600
Mean	329	54½	22	26½	53	80700

It will be shown in the following paper that the "free" water content of muscle, resting and fatigued, is about 0·77 g. per g. of muscle, the term "free" implying that the water is capable of dissolving in a normal manner substances added to it. Assuming the cations to be in free solution in this water, the

molal and equivalent concentrations corresponding to the above mean values are as follows :—

	K.	Na.	Ca.	Mg.	Total.
Molal concentration	0·109	0·031	0·007	0·014	0·161
Equivalent concentration	0·109	0·031	0·014	0·028	0·182

For the anions a similar calculation can be made with the following data :—

(*a*) *Chloride:* assuming the mean value given above, the molal and the equivalent concentrations are both 0·019.

(*b*) *Bicarbonate:* the bicarbonate content of fresh frog's muscle at *p*H 7·0, calculated from Stella's (1929) data, corresponds to 12 volumes p.c. of combined CO_2, or 0·007 molal.

(*c*) *Lactate:* assuming a resting value of 25 mg. per 100 g., the molal and the equivalent concentrations are 0·003.

(*d*) *Phosphate:* according to information kindly supplied by Mr. P. Eggleton (see also Eggleton and Eggleton (1929)) the phosphorus content of resting muscle may be taken as distributed as in Table II.

Table II.—Phosphorus Compounds in Resting Muscle.

Compound.	mg. P per 100 g.	Molal concentration.	No. of cations associated with one atom P at *p*H 7.	Equivalent concentration.	Reference.
Orthophosphate	18	0·008	1·64	0·013	Meyerhof and Suranyi (1926)
Pyrophosphate	30	0·006	1·05	0·013	Lohmann (1928*a*)
Creatine-phosphoric acid (phosphagen)	65	0·027	2·0	0·054	Meyerhof and Lohmann (1928)
Hexose monophosphoric ester	5	0·002	1·94	0·004	Meyerhof and Lohmann (1927)
Hexose diphosphoric ester	0	—	—	—	—
Other organic P (probably chiefly adenylic acid*)	15	0·006	1·9	0·011	Wassermeyer (1928)
Total	133	0·049	—	0·095	

* According to Lohmann (1929) a substance can be isolated from muscle, the Ba salt of which yields, on neutral hydrolysis, a mixture of pyrophosphate and adenylic acid. If the whole of the pyrophosphate and the adenylic acid fractions are combined in this way in the resting muscle, the sum of the molal concentrations must be diminished by 0·006; this does not seriously affect the following argument.

The titration curves from which the data of the third numerical column of Table II were obtained are to be found in the papers referred to in the last column. The column "equivalent concentration" gives the alkali equivalents required to bring the substance in question to pH 7·0.

The value of pH 7 is taken for resting muscle on the evidence: (*a*) of the experiments of Furusawa and Kerridge (1927) who, employing the glass electrode, found the pH of the resting skeletal muscles of cats to be 7·04 ± 0·03; (*b*) of the experiments of Meyerhof and Lohmann (1926) who, employing the hydrogen electrode on the NaCl extract of resting frog's muscle, found a mean value of 6·98; and (*c*) of the experiments of Stella (1929) on the CO_2 dissociation curve of resting frog's muscle, from which, at a partial pressure of CO_2 of 20 mm. Hg, the pH can be calculated by the Henderson-Hasselbalch equation as 6·9.

Fiske and Subbarow (1929), in a recent paper on phosphocreatine, referring to the amount of alkali combined with this substance at a given pH before and after hydrolysis, quote the experiments of Rous (1925), made by the method of intravital staining, as showing that the inside of the resting muscle must be as acid as 5·6. The application of the Henderson-Hasselbalch equation to Stella's (1929, p. 64) CO_2 dissociation curve of resting frog's muscle shows that to obtain a pH of 5·6 one would have to subject the tissue to a partial pressure of CO_2 of the order of two atmospheres. There may be minor corrections to apply to the Henderson-Hasselbalch equation when used for a complex heterogeneous system such as a living muscle is, but it cannot conceivably have errors of this magnitude; the partial pressure of CO_2 inside the living animal must be of the order of 20 mm., as Fenn found (1928), and not of two atmospheres. The value quoted by Fiske and Subbarow is obviously quite wrong. Chambers (1929) moreover, by the microinjection method, found the pH of the cytoplasm of a variety of amphibian cells, including muscle cells, to be 6·9 ± 0·1.

For the anions, therefore, of resting frog's muscle, we may summarise as follows:—

	Cl.	HCO_3.	Lactate.	Phosphate.	Total.
Molal concentration	0·019	0·007	0·003	0·049	0·078
Equivalent concentration ...	0·019	0·007	0·003	0·095	0·124

We have neglected the molal concentration of the protein anion; having a very high molecular weight its molal concentration is probably negligibly small.

The osmotic pressure of a resting frog's muscle freshly removed from the body has been shown above to be equal to that of a solution of 0·725 g. of NaCl in 100 g. of water; the molal concentration of the latter is 0·124. The sum of the molal concentrations of the ions present in muscle is 0·161 + 0·078 = 0·239. This is slightly less than the sum of the molal concentrations (0·124 + 0·124 = 0·248) of the ions of the isotonic NaCl solution (assumed fully dissociated). The deficit is almost exactly made up by free creatine, which has about the same molal concentration as orthophosphate, viz., 0·008 (Dulière, 1929). Thus the observed osmotic pressure of frog's muscle can be exactly accounted for if we assume that its known soluble constituents are in fact dissolved in the observed "free" water of the muscle. Only, however, by assuming *all* the known constituents to be so dissolved can a sufficient osmotic pressure be calculated. There is no latitude for supposing, for example, that an appreciable part of the K is combined, and not in free solution as a separate dissolved ion.

The sum of the equivalent concentrations of the cations is 0·182 N, of the anions 0·124 N. The deficit, 0·058 N is presumably accounted for by protein, existing in muscle as the ionised alkali-protein salt. Assuming 1 g. of muscle to contain 0·77 g. of "free" water and 0·16 g. of protein, the concentration of protein is 208 g. per 1000 g. of "free" water. If this provides the acid equivalent to the 0·058 N base, we may calculate that 1 g. of protein neutralises 28×10^{-5} g. equivalents of alkali at *p*H 7·0. According to the titration curves given by Cohn (1925), at *p*H 7 serum albumen, casein, egg albumen and gelatin combine respectively with 35×10^{-5}, 50×10^{-5}, 28×10^{-5} and 26×10^{-5} g. equivalents of alkali per g. of protein. The calculated value, therefore, for muscle protein is a very probable one.

According to Moore (1916) severely fatigued frog's muscle has a depression of freezing point 0·15° greater than that of resting muscle. This would make it isotonic, not with 0·725 p.c. NaCl, but with 0·983 p.c. The difference, 0·258 p.c., is not far from the 0·3 p.c. estimated by Cooke (1898). As long ago as 1865 Ranke published observations showing clearly—to put his findings in modern terms—a large increase in the osmotic pressure of fatigued muscles. These observations of Ranke are discussed below. The experiments described in the present paper show that the osmotic pressure of a muscle stimulated to extreme fatigue may rise by the equivalent of 0·35 p.c. NaCl. It is of interest therefore to make a calculation for fatigued muscle similar to that just given for resting muscle.

The *cations* and *chloride* are unaffected by fatigue. The *lactic acid* con-

centration reached is (say) 0·30 p.c.; assuming, as is shown in the following paper, that the "free" water present in 1 g. of fatigued muscle is 0·77 g., the molal concentration of lactate is 0·043. The *bicarbonate* content is reduced to a degree depending on the extent to which CO_2 is allowed to escape; this cannot be assessed without more closely defining the conditions. If we consider the case of an isolated muscle without oxygen rapidly fatigued the total CO_2, dissolved plus combined, will remain constant. We will assume the same value, viz., 0·007 M., as for the resting muscle. This is admittedly a rough estimate, but the whole amount is small. As regards phosphorus compounds, again on the advice of Mr. P. Eggleton, the distribution has been taken as in Table III.

Table III.—Phosphorus Compounds in Fatigued Muscle.

Compound.	mg. P per 100 g.	Molal concentration.	No. of cations associated with one atom P at *p*H 6·4.	Equivalent concentration.	Reference.
Orthophosphate	60	0·025	1·3	0·033	Meyerhof and Suranyi (1926)
Pyrophosphate*	30	0·006	0·83	0·010	Lohmann (1928*a*)
Creatine-phosphoric acid (phosphagen)	10	0·004	1·95	0·008	Meyerhof and Lohmann (1928)
Hexose monophoric ester	18	0·008	1·69	0·013	Meyerhof and Lohmann (1927)
Hexose diphosphoric ester	0	—	—	—	—
Other organic P (probably chiefly adenylic acid*)	15	0·006	1·50	0·009	Wassermeyer (1928)
Total	133	0·049	—	0·073	

* See footnote to Table II. According to Lohmann (1928*b*) a certain amount of the pyrophosphate present in muscle may be hydrolysed into orthophosphate as the result of long continued stimulation. If as much as one-third of the pyrophosphate were broken down in this way (each molecule becoming two) there would be an increase of molal concentration of 0·002. If the substance split were a compound of pyrophosphate and adenylic acid, each molecule broken down would become three and the increase of molal concentration would be 0·004. This again would not seriously affect the argument.

Details of this table are similar to those of Table II. The value *p*H 6·4 is taken for fatigued muscles on the evidence (*a*) of Furusawa and Kerridge (1927) who found a *p*H of 6·26 ± 0·07 by the glass electrode for the fatigued muscle of cats, and (*b*) of Meyerhof and Lohmann (1926) who in the water

extract of frog's muscle found a pH of about 6·3 (for 0·4 p.c. lactic acid) and of about 6·6 (for 0·25 p.c. lactic acid).

For the anions, then, we may summarise as follows :—

	Cl.	HCO_3.	Lactate.	Phosphate.	Total.
Molal concentration	0·019	0·007	0·043	0·049	0·118
Equivalent concentration	0·019	0·007	0·043	0·073	0·142

The sum, therefore, of the molal concentrations of the ions, positive and negative, present in fatigued muscle is $0 \cdot 161 + 0 \cdot 118 = 0 \cdot 279$. To this must be added, if we wish to calculate the osmotic pressure, the molal concentration of the creatine, viz., that originally present before fatigue 0·008 M., plus that formed from the phosphagen broken down 0·023 M., total 0·031 M. Thus the sum of the molal concentrations of all the known soluble constituents of fatigued muscle is 0·310 which is that of the ions of a 0·155 M. solution of NaCl. This is the same as one containing 0·906 g. of NaCl in 100 g. H_2O. It will be shown below that complete fatigue, leading to the formation of 0·3 p.c. lactic acid, causes an increase in osmotic pressure equivalent to that produced by adding about 0·35 g. of NaCl to 100 g. of Ringer's solution. This is considerably greater than the increase from 0·725 to 0·906 just calculated, viz., than 0·181. The increase, therefore, in the concentrations of the *known* constituents of muscle during fatigue is insufficient to account for the observed increase of osmotic pressure. The subject is further considered below.

The sum of the equivalent concentrations of the cations is 0·182 N, of the anions 0·142 N. The deficit, 0·040 N is presumably accounted for by protein anions. This deficit is less than that (0·058 N) calculated for the case of resting muscle, as is necessary since the pH has been assumed to change from pH 7·0 to pH 6·4. No stress can be laid upon the exact value calculated, since it depends upon the pH assumed for fatigued muscle : if another value, *e.g.*, pH 6·6, had been taken the alkali equivalent of the phosphorus would have been appreciably greater, the deficit less, and the change in protein combined with alkali greater. It is clear, however, that in stimulation to complete fatigue, a considerable part of the buffering is due to the "unionisation" of alkali protein. The neutralisation of acid cannot be attributed simply to phosphorus compounds, without assuming a pH for fatigued muscles (about 6·0) which is outside the range of actual observations.

Mammalian Muscle.—A calculation similar to the above, though not so complete or certain, can be made for the case of resting mammalian muscle. In the table on p. 82

of the paper by Katz (1896) are given the amounts, per 100 g. of fresh tissue, of K, Na, Ca, Mg, Cl, total P and water, present in the muscles of ox, calf, deer, rabbit, dog and cat. These quantities have been averaged. The mean water content is 0·76 g. per g. of muscle. Arguing on the analogy of frog's muscle the "free" water content is presumably about 0·71 g. per g. of muscle. The average concentrations have been expressed in g. mols. (or g. ions) per 1000 g. of "free" water. The relative distribution of soluble P has been assumed to be the same as in the case of resting frog's muscle, in which (with 77 p.c. "free" water) 133 mg. P per 100 g. of muscle was calculated to give a molal concentration of 0·049. With a "free" water content of 71 p.c. the molal concentration, for a given quantity of P per g. of muscle, will be greater in the ratio of 77 : 71.

	K.	Na.	Ca.	Mg.
Concentration, g. per 100 g. muscle	0·3648	0·0724	0·0100	0·0275
Molal concentration	0·132	0·044	0·003	0·016

	Cl.	Soluble P.	HCO_3.	Lactate.
Concentration, g. per 100 g. muscle	0·0588	0·1960	*	0·015
Molal concentration	0·023	0·078	0·016	0·002

* Assumed to be equivalent to 0·25 c.c. of CO_2 per g. of muscle.

The sum of the molal concentrations of all the ions is 0·314. To this, if we wish to calculate the osmotic pressure, must be added the free creatine, which—for lack of better knowledge—we will assume to have the same molal concentration, 0·008, as in resting frog's muscle. The total, 0·322, is equal to the sum of the molal concentrations of the ions of 0·161 M NaCl which is 0·94 g. of NaCl to 100 g. of H_2O. The freezing point of human blood corresponds to 0·96 g. of NaCl in 100 g. of water, and muscle is presumably isotonic with blood. Clearly the osmotic pressure of mammalian muscle can be explained approximately—so far as present evidence goes—by the hypothesis that *all* its soluble constituents are in solution in a quantity of "free" water equal to about 71 p.c. of its weight. Again there is no latitude for supposing that any considerable part of the soluble constituents is not in fact dissolved.

IV. The Change of Vapour Pressure in Stimulated Muscle.

(*a*) *The Complete Absence of an Increment in Resting Heat-rate after Anaerobic Stimulation, when Condensation of Water Vapour is prevented.*

That a decrease of vapour pressure and the resulting condensation of water on the muscle are, in fact, the cause of the increase in resting heat-rate observed after anaerobic stimulation, is shown by the following simple and decisive experiment, in which condensation was prevented by filling the thermopile chamber with liquid paraffin instead of nitrogen.

A symmetrical thermopile, the hot and cold junctions being on opposite

faces, was insulated with "Elo" and shellac and used for a single frog's sartorius, which lay on one face. Employed in nitrogen this instrument showed the usual increment in heat-rate after stimulating the muscle. Several experiments were performed in which, after a preliminary soaking of several hours in Ringer's solution to render it permanently excitable in the sense of Dulière and Horton (1929), a muscle was placed in paraffin oil ("Regelax" by Messrs. Boots) on this thermopile. Pure nitrogen was passed for about an hour through the chamber containing oil and muscle, to assist in temperature equalisation and to remove oxygen from the oil. The resting heat-rate was read. The muscle was then stimulated by a series of induction shocks to fatigue, responding very well and liberating a large amount of heat. After this heat had been dissipated the resting heat-rate was found to be the same as before. There was no sign of an increment in heat rate as the result of anaerobic activity. The *paraffin* oil seemed to have no harmful effect on the muscles, in fact in these experiments they behaved rather better than usual. Thus, *if condensation of water on the muscle be prevented, the increment in heat-rate due to anaerobic activity does not appear.*

The use of paraffin oil in a thermopile chamber was tried previously by Hartree and Hill (1920, p. 93), but they found that, on the whole, muscles did not survive so well in paraffin as in a gas. This is now known to be due to the fact that, when paraffin was used in the chamber, the muscle had not been subjected to a preliminary soaking in Ringer's solution, and consequently tended to become reversibly inexcitable (Dulière and Horton, 1929). A muscle, given a sufficient preliminary soaking in Ringer's solution before mounting on the thermopile, behaves in paraffin at least as well as in a gas; and the use of paraffin has several advantages (*a*) in avoiding the effects of changes of vapour pressure, (*b*) in improving electrical insulation, and (*c*) in increasing zero-stability, at least with a symmetrical thermopile. Moreover the high solubility of oxygen in paraffin oil makes it possible to carry out experiments on the recovery heat production in that medium, of which there are several advantages.

(*b*) *The Relation between the Change of Vapour Pressure and the Degree of Fatigue.*

It was stated by Hill (1928*b*) that a linear relation exists between the increment in heat-rate, due to stimulation of a muscle in nitrogen, and the amount of heat liberated as the result of activity. The increment in heat-rate has been found to be a measure of the decrease of vapour pressure and therefore of the increase of osmotic pressure, or of total molal concentration. Thus the number of molecules (or ions) liberated in anaerobic activity should be proportional to the heat set free. It seemed important to test this simple relation by further experiments.

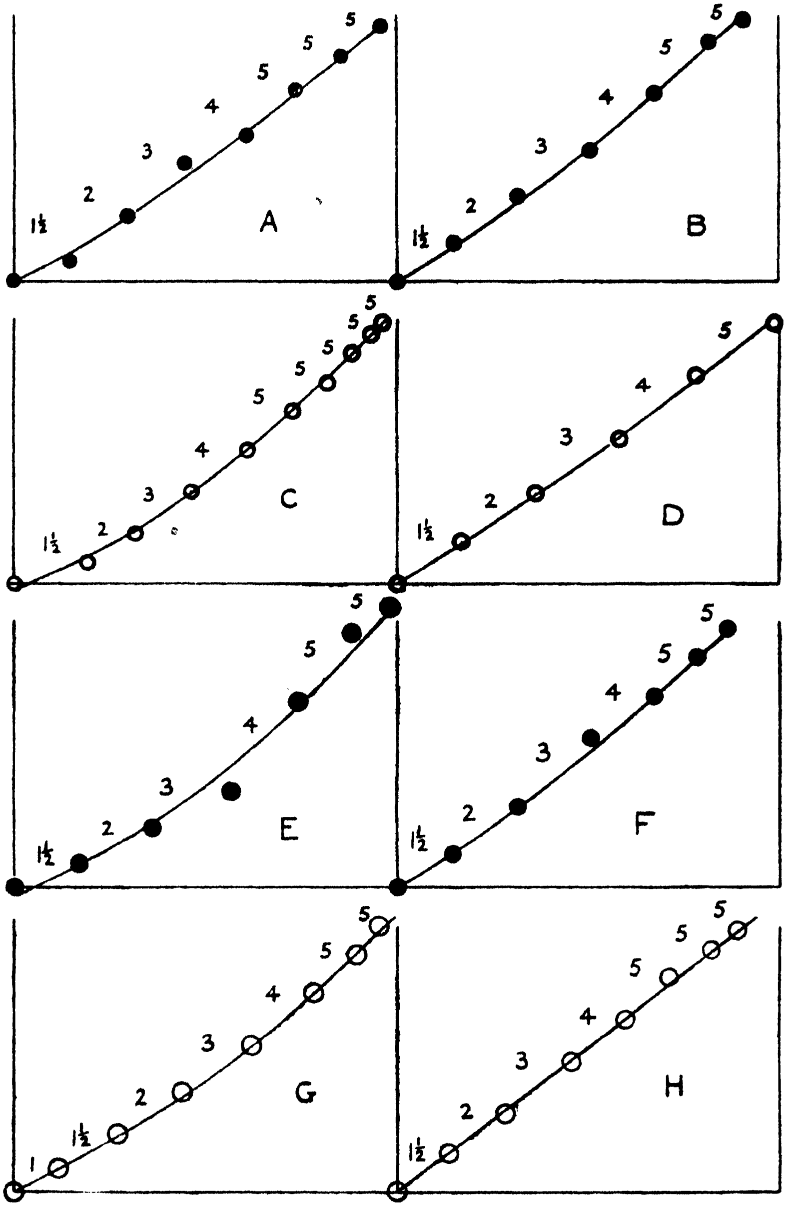
A muscle was soaked in Ringer's solution on the thermopile for an hour or two. The solution was replaced by pure nitrogen,* and a steady reading was obtained on the scale, representing the true (metabolic) rate of heat production at rest. A series of maximal tetanic stimuli was then applied, the heat liberated as the result of each stimulus being read on the scale ; the muscle after each stimulus was allowed to cool, and the rate of resting heat production again recorded. Eight experiments were performed, all the results being shown in the accompanying diagram.

Each point represents a pair of observations, the abscissa being the total heat set free by stimulation, up to and including the stimulus in question, the ordinate the total increment in resting heat-rate determined after the heat produced by the last stimulus had been dissipated. The durations of the successive stimuli are shown in seconds above each curve. In most cases the muscle finished in a state of considerable fatigue ; the last stimulus of 5 seconds produced considerably less heat than the first stimulus of $1\frac{1}{2}$ seconds. The units of heat and of heat-rate are arbitrary ; no calibration was carried out. The temperature lay between 19·3° and 20·5° C.

It is evident that an approximately linear relationship does exist, but in every case there is a tendency for the curve to be more or less concave upwards. The total molal concentration therefore of the muscle tends to increase rather more than in direct proportion to the total heat set free. The major chemical changes known to occur are lactic acid formation and phosphagen breakdown. The heat produced per gramme of lactic acid set free is given by Meyerhof and Suranyi (1929) as 380 to 390 calories. These authors note that their mean value was obtained with a 0·15 to 0·25 p.c. lactic acid formation ; with smaller amounts of lactic acid they found a rather higher value. Thus for a given amount of heat the increase in lactic acid concentration is less in the earlier than in the later stages of fatigue. Prof. Meyerhof informs us that the caloric quotient in complete exhaustion is certainly not as high as 385 ; he would estimate it as being at the most 350. If lactic acid formation were the only process concerned this progressive fall in its caloric quotient during the gradual onset of fatigue would give the observed upward tendency to the curves in the figure.

The matter, however, is complicated by the breakdown of phosphagen. According to Nachmansohn (1929), if a muscle be subjected to a series of 2 sec. tetani the chief breakdown of phosphagen occurs in the first two or three contractions, after which any further change becomes very gradual. There is

* Freed from oxygen by passing over heated copper in a quartz tube.



The results of eight experiments, in which the increment in resting heat-rate of a muscle in nitrogen, due to a series of maximal tetani, is plotted (as ordinate) against the total heat set free by stimulation (as abscissa). The units of heat and of heat-rate are arbitrary. The durations of successive stimuli are shown in seconds along each curve. The muscle in every case finished appreciably (*e.g.*, A, D) or very (*e.g.*, C, H) fatigued as shown by the amount of heat set free in the last contraction (5 seconds) as compared with the first ($1\frac{1}{2}$ seconds). Temperatures between 19·3° and 20·5° C.

no sign in the figure of an excessive production of new molecules in the first few contractions ; indeed, so far as there is any departure from a linear relation between molecules (or ions) liberated and heat produced, the departure is in the opposite direction.

As regards other possible breakdowns, Lohmann (1928*b*) has shown that orthophosphate appears at the expense of pyrophosphate, particularly in the later stages of exhaustion. The total increase of molal concentration thereby caused is not large, though it is twice as great if we suppose that an adenylic acid-pyrophosphate complex is split (see Lohmann, 1929). The concentration of this breakdown into the later stages of exhaustion would give an upward tendency to the curves of the figure.

(*c*) *The Absolute Value of the Change of Vapour Pressure produced by Anaerobic Activity of Muscle.*

For a given thermopile, chamber and muscle, if p be the vapour pressure of the muscle and p' the vapour pressure of the fluid on the instrument and on the walls of the chamber, the rate of condensation of water on the muscle, and therefore the rate of heat production in excess of the true metabolic rate, must be directly proportional to $(p' - p)$. A given change, therefore, in the value of p should produce the same effect as an equal and opposite change in p'. Hence the increment in heat-rate due to a degree of anaerobic activity which produced a fall a in the vapour pressure p of the muscle, must be the same as the increment in heat-rate due to a rise a in the vapour pressure p' of the chamber. Thus the instrument can be calibrated to read the change of vapour pressure of the muscle by washing it out with a solution, the vapour pressure of which differs by a known amount from that of the solution originally in it.

Various attempts were made, but without success, to introduce nitrogen saturated with the water vapour of a given solution into an otherwise dry thermopile chamber. Even with the most careful regulation of the temperature, and with slow passage of the gas over a long column of glass beads wet with the solution, standing in the same thermostat as the instrument, very irregular readings were obtained. It seems to be well nigh impossible to ensure complete saturation of the gas in this way. This indeed is not difficult to understand. It will be shown below that the decrease of vapour pressure due to the complete fatigue of a frog's sartorius is about equal to that caused by adding 0·35 p.c. of NaCl to Ringer's solution. Assuming a relative molal depression of vapour pressure of 0·0330, this means that complete fatigue causes a change of vapour

pressure of only about 2 parts per thousand ; if the gas came into the chamber 99·9 p.c. instead of fully saturated with moisture, there would be an error of 50 p.c. in the reading. To reduce the error to 1 p.c. would require the saturation to be 99·998 p.c. complete. To attain to such a degree of saturation it appears to be necessary that the instrument, and the walls of the chamber, should be moist with the given solution; then, and then only, does the gas become sufficiently saturated to allow consistent and reliable readings to be obtained.

A difficulty arises in this connection, viz., that it is not possible to wash the instrument and chamber with a new solution, without, at the same time, washing the muscle. In order, as far as possible, to avoid changes in the muscle itself due to washing with a different solution it is essential that the period of washing should be very short. The test solutions therefore were run in for a very few seconds, stirred by blowing, and then rapidly withdrawn. In this way a minimal change is produced in the muscle itself; the chamber and instrument, however, are brought accurately to the vapour pressure of the new solution. It is true that a film of the new solution is left on the surface of the muscle; this, however, when the bulk of the fluid is withdrawn, will rapidly attain the same osmotic pressure as the interior of the muscle, by the diffusion of water in or out; the net effect after a few minutes will be simply a slight dilution of the muscle (if the solution was hypotonic) or a slight concentration (if the solution was hypertonic). Compared with the total weight of the muscle the film left on its surface is not large, and the resulting change in its concentration must be small. The error, moreover, can be almost completely avoided by adopting the procedure described below.

The experiments were performed as follows:—A sartorius muscle was left to soak in Ringer's solution on a thermopile in its chamber at a temperature of about 19° C., for a time sufficient to allow it to attain diffusion equilibrium with the solution. This was then replaced by oxygen and the resting heat-rate recorded; let this be h mm. on the scale. Two stock solutions had been prepared, containing the usual constituents of Ringer's solution, but in 60 p.c. and 140 p.c. respectively of the usual concentration; they will be referred to as 0·6 R and 1·4 R. In the majority of experiments the Ringer's solution was isotonic with 0·671 p.c. NaCl, so these solutions were isotonic with 0·403 p.c. and 0·940 p.c. NaCl respectively. A 100 c.c. pipette was filled with 1·4 R solution, very carefully adjusted to the temperature of the thermostat, so that its introduction should produce a minimal disturbance. This was forced into the chamber, stirred by blowing and rapidly withdrawn; oxygen was then

run in as before. Within half an hour a steady reading $-H_1$ for the resting heat-rate was obtained, negative in sign owing to the fact that the vapour pressure of muscle is greater than that of 1·4 R; consequently the muscle evaporates, and the negative heat of evaporation masks the positive heat production of metabolism.

A 100-c.c. pipette was then filled with 0·6 R solution, again carefully adjusted to the temperature of the thermostat. This was rapidly introduced and withdrawn; oxygen was then run in. Within half an hour a steady reading $+H_2$ for the resting heat-rate was obtained, the true metabolic heat-rate h being added to the heat of condensation of water on the muscle, which now had a lower vapour pressure than the solution in the chamber. The difference $(H_2 - h)$ was taken to correspond (see below) to the difference of vapour pressure between 1·0 R and 0·6 R.

The observations were then repeated with 1·4 R and with 0·6 R, thus giving a second value for $(H_2 - h)$. The muscle was then washed in normal Ringer's solution (1·0 R) for an hour; the solution was replaced by nitrogen; the resting heat rate was recorded; the muscle was stimulated to fatigue by a series of shocks, its total heat-production being measured; the resting heat rate in nitrogen was again recorded, the increment I in heat rate being obtained by subtraction; the muscle was then killed; an electrical calibration was carried out, in order to express in absolute units the heat liberated as the result of stimulation; and finally the muscle was removed and weighed.

The change in vapour pressure of the muscle was expressed in terms of the corresponding change in the concentration of a NaCl solution. Suppose that $(H_2 - h)$ mm. of the galvanometer scale correspond to a change from 1·0 R to 0·6 R, *i.e.*, to a change equivalent to 0·268 p.c. NaCl. Then 1 mm. on the scale is equivalent to $0{\cdot}268/(H_2 - h)$ p.c. NaCl. Thus the increment I in heat rate, due to stimulation, corresponds to an increase of $0{\cdot}268I/(H_2 - h)$ p.c. NaCl. This was compared with the lactic acid produced, as calculated from the heat liberated during stimulation, employing a caloric quotient of 350 calories per gramme of lactic acid.

The reason for adopting the procedure described above is that any concentration of the muscle by the film of stronger solution left behind after washing out the chamber with 1·4 R is exactly balanced by the dilution of the muscle by the film of weaker solution left behind after washing out with 0·6 R; so that the muscle, after the second washing, is exactly in its initial state, while the chamber is saturated with the vapour of 0·6 R. We thus obtain a true value for the change of vapour pressure in passing from 1·0 R to 0·6 R.

The cause of this is best shown by a numerical example. Let us assume that a muscle of weight 1, in equilibrium with 1·0 R, is washed for a few seconds with 1·4 R, and that a

film of the latter solution of weight 0·1 is left on its surface. By a process of osmosis the water of the muscle rapidly passes out and dilutes the film, an equilibrium being reached in which the concentration of each is $(1\cdot 0 + 0\cdot 14)/1\cdot 1 = 1\cdot 036$ R. Thus the difference $(h + H_2)$ corresponds not to (1·4 R − 1·0 R) but to (1·4 R − 1·036 R); it is 9 p.c. too low. The muscle, moreover, has lost, by osmosis into the film of solution, the amount of water required to bring a quantity 0·1 of 1·4 R to a concentration of 1·036 R; the final weight of the muscle therefore is 0·964.

Let us now wash the muscle rapidly with 0·6 R, and assume that a film of that solution of weight 0·1 is left on its surface. By a process of osmosis the water of the film rapidly passes into the muscle and leaves the film more concentrated, an equilibrium being reached in which the concentration of each is $(0\cdot 964 \times 1\cdot 036 + 0\cdot 1 \times 0\cdot 6)$ R$/(0\cdot 964 + 0\cdot 1)$ which is equal to 0·996 R. The final weight of the muscle is 1·004. Thus after the second washing the muscle has returned almost exactly to its initial state, and the reading of the heat rate $(H_2 - h)$ should correspond within 1 p.c. to the difference of vapour pressure between 1·0 R and 0·6 R.

Similarly it may be shown that any error caused by osmosis of water, or diffusion of electrolytes, *during* the few seconds of washing with 1·4 R, will be almost exactly reversed by the subsequent washing with 0·6 R. Hence the quantity $(H_2 - h)$ may be regarded as an accurate measure of the vapour pressure difference between the solution 0·6 R and a muscle in equilibrium with 1·0 R.

A small correction must be applied for the dilution of one solution by the remains of the preceding solution. In the case of one thermopile and chamber the difference between 1·0 R and 0·6 R must, for this reason, be taken actually as 0·335 R, with another thermopile and chamber, as 0·392 R.

A description of a typical experiment follows:—

Experiment of 14.10.29.—Sartorius of *R. temp.* dissected 9.10 a.m., at 9.45 a.m. in chamber in oxygenated Ringer's solution (1·0 R). At 11 a.m. 1·0 R out, oxygen in. At 11.35 a.m. resting heat-rate $h = 39$ mm. on scale. 1·4 R introduced and withdrawn; at 12.5 p.m. $H_1 = -49\frac{1}{2}$ mm. 0·6 R introduced and withdrawn; at 12.35 p.m. $H_2 = +157$ mm. 1·4 R introduced and withdrawn; at 1·06 p.m. $H_1 = -62$. 0·6 R introduced and withdrawn; at 1.36 p.m. $H_2 = +162$. 1·4 R introduced and withdrawn; at 2·04 p.m. $H_1 = -58$. 0·6 R introduced and withdrawn; at 2.35 p.m. $H_2 = +140$.

Muscle now washed in 1·0 R till 3.03 p.m., then in N_2. Heat-rate in N_2 before stimulation 27 mm. Stimulated to exhaustion by induction shocks 42 per minute. Total heat determined from the area of the deflection-time curve, and subsequent calibration as in (Hill, 1928*a*), 1·11 cal. per g., which is equivalent to 0·317 p.c. lactic acid produced, assuming a caloric quotient of 350. Heat-rate in N_2 after stimulation 177 mm. Increment in heat-rate $I = 150$ mm.

Calculation—

$h = 39$ mm. on scale.

$H_2 = 157, 162, 140$, mean 153 mm.

$H_2 - h = 114$ mm.

Thus 114 mm. ≡ 0·392 R ≡ 0·263 p.c. NaCl.

Increment in heat-rate 150 mm., therefore ≡ 0·346 p.c. NaCl.

Lactic acid calculated 0·317 p.c.

The production therefore of one part of lactic acid is accompanied by a change of vapour pressure corresponding to $0\cdot 346/0\cdot 317 = 1\cdot 09$ parts of NaCl.

The results of eight experiments are summarised in Table IV.

Table IV.—Increase of Osmotic Pressure in Fatigued Muscles.

Experiment of 14.10.29. $h = 39$; $H_1 = -49, -62, -58$; $H_2 = +157, 162, 140$, mean 153.

0·392 R ≡ 0·263 p.c. NaCl ≡ 114 mm.

Increment I due to stimulation = 150 mm. ≡ 0·346 p.c. NaCl.

Heat 1·11 cal. per g. ≡ 0·317 p.c. lactic acid.

1 part lactic acid ≡ 1·09 parts NaCl.

Experiment of 15.10.29. $h = 60$; $H_1 = -52, -56$; $H_2 = 184, 182$, mean 183.

0·392 R ≡ 0·263 p.c. NaCl ≡ 123 mm.

Increment I = 162 mm. ≡ 0·347 p.c. NaCl.

Heat 1·023 cal. per g. ≡ 0·293 p.c. lactic acid.

1 part lactic acid ≡ 1·18 parts NaCl.

Experiment of 18.10.29. $h = 75$; $H_1 = -108, -100$; $H_2 = 256, 282$, mean 269.

0·392 R ≡ 0·263 p.c. NaCl ≡ 194 mm.

Increment I = 275 mm. ≡ 0·373 p.c. NaCl.

Heat 1·066 cal. per g. ≡ 0·305 p.c. lactic acid.

1 part lactic acid ≡ 1·22 parts NaCl.

Experiment of 8.11.29. $h = 56$; $H_1 = -32, -37$; $H_2 = 150, 134$, mean 142.

0·335 R ≡ 0·225 p.c. NaCl ≡ 86 mm.

Increment I = 150 mm. ≡ 0·392 p.c. NaCl.

Heat 1·165 cal. per g. ≡ 0·333 p.c. lactic acid.

1 part lactic acid ≡ 1·18 parts NaCl.

Experiment of 11.11.29 (nitrogen). $h = 7$; $H_1 = -67, -75$; $H_2 = 100, 90$, mean 95.

0·335 R ≡ 0·225 p.c. NaCl ≡ 88 mm.

Increment I = 91 mm. ≡ 0·233 p.c. NaCl.

Heat 0·994 cal. per g. ≡ 0·284 p.c. lactic acid

1 part lactic acid ≡ 0·82 parts NaCl.

Experiment of 13.11.29. $h = 30$; $H_1 = -57, -50$ $H_2 = 112, 106$, mean 109.

0·335 R ≡ 0·225 p.c. NaCl ≡ 79 mm.

Increment I = 109½ mm. ≡ 0·312 p.c. NaCl.

Heat 1·058 cal. per g. ≡ 0·302 p.c. lactic acid.

1 part lactic acid ≡ 1·03 parts NaCl.

Experiments of 11.2.30. In these experiments the Ringer's solution was isotonic with 0·703 p.c. NaCl and no dilution occurred in the chamber when substituting one solution for another. The solutions were kept in bottles in the thermostat bath until required and then rapidly transferred to the muscle chamber. The solutions employed, 0·7 R and 1·3 R, were used in that order.

(A) 0·3 R ≡ 0·211 p.c. NaCl ≡ 96½ mm.

Increment I = 124 mm. ≡ 0·271 p.c. NaCl.

Heat 0·737 cal. per g. ≡ 0·210 p.c. lactic acid.

1 part lactic acid ≡ 1·29 parts NaCl.

(B) 0·3 R ≡ 0·211 p.c. NaCl ≡ 116 mm.

Increment I = 117½ mm. ≡ 0·214 p.c. NaCl.

Heat 0·621 cal. per g. ≡ 0·177 p.c. lactic acid.

1 part lactic acid ≡ 1·21 parts NaCl.

The increment I in heat-rate due to fatigue in nitrogen is expressed in Table IV in terms of the increment in NaCl concentration which would produce the same fall of vapour pressure in Ringer's solution. Now the increment in heat-rate has been shown already to be very nearly proportional to the total heat set free in activity, and hence presumably in normal muscles to the lactic acid produced. In order therefore to compare one experiment with another it is necessary to reduce them to some standard conditions; we have compared them for equal quantities of lactic acid set free per g. of muscle as calculated from the heat. For one part by weight of lactic acid set free in the muscle by stimulation, the change of vapour pressure corresponds, in the eight experiments of Table IV, to the following parts by weight of NaCl added to Ringer's solution:—1·09, 1·18, 1·22, 1·18, 0·82, 1·03, 1·29, 1·21; mean (omitting 0·82) 1·17. Taking 0·3 p.c. as a typical value for the lactic acid concentration in an exhausted muscle, the change of vapour pressure is equivalent to that caused by adding $1 \cdot 17 \times 0 \cdot 3 = 0 \cdot 35$ p.c. of NaCl to Ringer's solution.

Moore (1916) found an extra depression of freezing point of 0·15° C. on fatiguing his muscles. This corresponds, as shown above, to 0·258 p.c. of NaCl added to Ringer's solution, and (taking our mean value 1·17) to 0·22 p.c. of lactic acid produced by the muscles of his American bull frogs. This is a very probable value.

One gramme of lactic acid in 1000 g. of muscle is 0·01445 M., reckoned in the "free" water (77 p.c.) of the muscle. The addition of 1·17 g. of NaCl to 1000 g. of Ringer's solution raises the concentration by 0·0202 M., or if we consider the sum of the molal concentrations of the two ions, by 0·0404. This is 2·8 times as great as the molal concentration of the lactate ions set free. *The increase therefore in the osmotic pressure of muscle on stimulation to fatigue is 2·8 times as great as the osmotic pressure of the lactate ions liberated.*

To explain the discrepancy it might be argued that the lactate ion is necessarily accompanied by an alkali ion, say K, and that before combination with lactate the K-ion was not free in solution to affect the osmotic pressure. If this were the case the increase of osmotic pressure on stimulation to fatigue would be 1·4 times as great as that of the lactate and K-ions liberated. With the aid of the phosphate changes known to occur, and the creatine set free, it would then be possible to give a complete explanation of the observed increase of osmotic pressure. There is, however, grave difficulty in assuming that the K-ions associated with the lactate ions after stimulation were not free in solution before stimulation. The existence of electrically undissociated compounds of K in muscle is unlikely, and even if the anions were unable to diffuse

(for example, if they were part of the protein structure of the muscle) the cations would be free to change partners and so to move about and presumably to affect the osmotic pressure. Moreover we have shown above that the osmotic pressure of resting muscle can be calculated fairly exactly from the known soluble constituents dissolved in the known "free" water of the muscle. If we were to argue that the cations before stimulation were not free to affect the osmotic pressure, we should be left with a serious deficit in calculating the osmotic pressure of resting muscle. Moreover, it will be shown below that an acid (CO_2) added artificially to resting muscle causes an increase of osmotic pressure approximately equal to that of its anions alone, certainly not twice as great. It is clear, therefore, that the cations play no part in the phenomenon considered.

It seemed possible that a decrease of the "free" water, associated with fatigue, might be one cause of the excessive increase of osmotic pressure. If the various soluble substances present in muscle were confined to a smaller amount of water their osmotic pressure would rise. For example, if the "free" water, in 1 g. of resting muscle were 0·77 g., and in 1 g. of fatigued muscle 0·60 g. the osmotic pressure, equivalent in resting muscle to that of 0·70 p.c. NaCl, would rise in fatigued muscle to that of 0·90 p.c. NaCl, *without the liberation of any new ions or molecules.* This attractive way of accounting for the large increase of osmotic pressure is unfortunately negatived by the fact that in fatigued muscle the "free" water appears to be precisely the same as in resting muscle, as is shown in the following paper.

In fatigue the formation of lactic acid is accompanied by the liberation of creatine through the breakdown of phosphagen. The phosphagen content of fresh muscle was given above (Table II) as 0·027 M., of fatigued muscle (Table III) as 0·004 M. The difference 0·023 M. represents the creatine set free. The production of 0·3 p.c. of lactic acid represents an additional 0·043 M. Together lactate and creatine account for 0·043 + 0·023 = 0·066 M., which is the sum of the molal concentrations of the ions of 0·193 g. of NaCl in 100 g. of H_2O. But we have just seen that this degree of fatigue causes a change of osmotic pressure equal to that of 0·35 p.c. of NaCl added to Ringer's solution. *Thus the increase in the osmotic pressure of muscle on stimulation to fatigue is* 1·81 *times as great as the osmotic pressure of the creatine molecules and the lactate ions produced.*

It is possible that in the resting muscle creatine phosphoric acid is part of some more complex molecule and so exerts no osmotic pressure of its own. In that case the breakdown of phosphagen into free creatine and phosphate

would liberate twice as many new molecules as we have calculated, the sum of whose molal concentrations would be 0·046 instead of 0·023. This would give 0·089 M. as the increase accounted for by lactate formation and phosphagen breakdown together; which is equivalent to the sum of the molal concentrations of the ions of 0·26 g. of NaCl in 100 g. of H_2O. *This is about* 3/4 *of the increase of osmotic pressure actually observed.*

Two other changes contributing to the rise of osmotic pressure must be considered, the liberation of ammonia and the splitting of pyrophosphate. According to Parnas and Mozolowski (1927) the ammonia content of muscle increases, in severe fatigue, by an amount corresponding to about 0·002 p.c. of N. Calculated in the " free " water of muscle this is about 0·002 M. According to Lohmann (1928*b*), in exhausted muscle a certain amount, say, one-third, of the pyrophosphate is hydrolysed into orthophosphate; if this pyrophosphate had previously been combined with adenylic acid (Lohmann, 1929) each molecule split would become three, and the increase in molal concentration (see Table III above) would be 0·004. Taking account, therefore, of every known possibility, and making a maximal allowance in each case, the increase in total molal concentration as the result of fatigue can be calculated as follows:—

Lactate (0·3 p.c.)	0·043 M.
Creatine	0·023 M.
Phosphate	0·023 M.
Ammonia	0·002 M.
Pyrophosphate-adenylic acid	0·004 M.
Total	0·095 M.

Now 0·095 M is the sum of the molal concentrations of the ions of a solution of 0·28 g. of NaCl in 100 g. of water. This is still only 80 p.c. of 0·35, the observed change. Thus all the reactions already known to occur in fatigue are insufficient, even taking the most favourable view of them, to account for the change of osmotic pressure actually observed. Presumably, therefore, some other changes, at present unsuspected, are involved. It is indeed unlikely that, after the striking progress of the last few years, we should at this particular moment have discovered all the reactions involved in muscular activity.

The increase of osmotic pressure of muscles in rigor has been measured, as described in the following paper, by another method, as an incident in the determination of the " free " water. The mean value of the osmotic change in rigor is equivalent to that of 0·45 p.c. NaCl added to Ringer's solution.

(d) The Change of Vapour Pressure caused by the Neutralisation of Carbonic Acid in Resting Muscle.

We discussed above the question of whether the osmotic effect of the K-ions should be added to that of the lactate ions liberated during activity, or whether the K-ions ought to be regarded as being free before the lactic acid was formed. It seemed desirable, therefore, to find out whether the artificial liberation of an acid in muscle, and its neutralisation by the alkali there present, would produce an osmotic effect equal to that of the anions alone, or of the anions and their associated cations together. It was necessary to use a volatile acid, since soaking the muscle in a solution inevitably brings it into osmotic equilibrium with the latter, whatever reactions take place inside the tissue. The obvious acid to choose was carbonic, partly because it is a natural constituent of muscle and penetrates very easily, and partly because it has already been investigated by Hill (1928*b*) and Stella (1929).

If a muscle on a thermopile, previously soaked in Ringer's solution, and exhibiting its true (metabolic) resting heat rate in nitrogen, be subjected to an atmosphere of CO_2, there is a large liberation of heat, complete within an hour or less, due to the solution and combination of CO_2. According to Stella (1929) a resting frog's sartorius takes up in combination about 47 vols. per cent. of CO_2 when subjected to an atmosphere of that gas; this represents a 0·027 M. solution of bicarbonate, calculated for the "free" water (77 p.c.) of muscle.

If the anion were the only new one, the bicarbonate combining with pre-existing alkali ions, the resulting change in osmotic pressure would be equivalent to that due to adding 0·0135 M. = 0·079 p.c. NaCl to Ringer's solution. If, however, the bicarbonate ion captured a K-ion previously unable to affect the osmotic pressure, the resulting change would correspond to adding 0·158 p.c. NaCl to Ringer's solution. It will be noticed that we have neglected the effect of dissolved CO_2; the physical solution of CO_2, as distinguished from its combination, must affect the vapour pressure of the solution in the chamber and of the muscle to almost precisely the same extent, so that any difference observed must be due to the combined and not to the dissolved CO_2. Owing to the absence of bicarbonate and phosphate from the Ringer's solution, there was no combined CO_2 in the latter.

In a previous paper (Hill, 1928*b*) it was stated that no increment in heat-rate occurs when a resting muscle is placed in CO_2. Certainly the increment is very small compared with that due to stimulation in nitrogen. With sufficient

precautions, however, it can be measured. One reason why it is so small is—as we have found—that a given difference of vapour pressure causes a smaller rate of condensation, or evaporation, in CO_2 than in nitrogen or air. The causes of this are (*a*) the lower diffusion constant of water-vapour molecules through CO_2, and (*b*) the greater viscosity and density of that gas, hindering the carriage of water molecules by convection from the evaporating to the condensing surface.

The chief precaution required in these experiments is to soak the muscle for a very long time in Ringer's solution before reading its resting heat-rate in nitrogen. The difference to be observed between nitrogen and CO_2 is small, and it is necessary to ensure that the resting heat-rate in nitrogen represents only the true metabolic heat-rate of the muscle. *This is assumed to be the same in nitrogen and in* CO_2. When the reading h_1 in nitrogen had been obtained CO_2 was introduced, and after a sufficient interval to allow the muscle to come into equilibrium with that gas the heat-rate h_2 in CO_2 was read. The difference* $(h_2 - h_1)$ represents the increment in heat-rate due to the change of vapour pressure caused by the combination of carbonic acid with the muscle.

It was now necessary, as before, to wash the apparatus and chamber with the solutions 1·4 R and 0·6 R, in order to determine the value of a given scale deflection in terms of vapour-pressure difference. The procedure was exactly as described above, except that great care was exercised to allow no air to enter, but only CO_2, on withdrawing the liquid after the rapid washing out of the chamber. The difference $(H_2 - h_2)$ was used, as before, as a measure of the vapour-pressure difference between 1·0 R and 0·6 R.

Details of nine experiments are given in Table V. The agreement between different experiments is not very good, owing to the difficulty of determining the small difference $(h_2 - h_1)$ with sufficient accuracy. The mean, however, of the nine experiments is probably not far from the truth. The same thermopile was used throughout.

The mean value, therefore, of the change produced in the vapour pressure of the muscle by allowing its buffers to come into equilibrium with CO_2 at 1 atmosphere pressure (neglecting the effect of physically dissolved CO_2, as described above) is equivalent to that produced in Ringer's solution by adding 0·069 p.c. NaCl. This is slightly less than the 0·079 p.c. calculated above

* To be able to take this difference directly without separate calibration of the apparatus in nitrogen and CO_2 requires that the calibration numbers should be the same in the two cases. We have carefully verified this and found an exact equality.

Table V.—The Change of Vapour Pressure due to the Combination of CO_2 with Muscle.

h_1 means the true metabolic resting heat-rate in nitrogen at the beginning of the experiment.

h_2 the rate observed after attaining equilibrium in CO_2.

H_2 the heat-rate in CO_2 after washing with 0·6 R.

E represents the osmotic change produced by CO_2 in terms of the equivalent addition of NaCl to Ringer's solution.

$$E = \frac{h_2 - h_1}{H_2 - h_2} \times [\text{NaCl equivalent of difference between } 1\cdot0 \text{ R and } 0\cdot6 \text{ R}].$$

In the first seven experiments, after allowance for dilution, the NaCl equivalent of the difference between 1·0 R and 0·6 R was 0·225 p.c.; in the last two experiments 0·268 p.c. A more sensitive galvanometer was used in the last two experiments.

Date.	h_1.	h_2.	$h_2 - h_1$.	H_2.	H_2 (mean).	$H_2 - h_2$.	E. NaCl p.c.
30.10.29	20	32½	12½	82, 85	83½	51	0·055
1.11.29	19½	48	28½	118, 108	113	65	0·099
4.11.29	24½	54	29½	124, 126	125	71	0·094
5.11.29	23	43	20	107, 132	120	77	0·059
6.11.29	29	49	20	118, 134	126	77	0·059
7.11.29	30½	45½	15	129½	129½	84	0·044
7.11.29	20½	53	32½	132	132	79	0·092
–.12.29	29	77	48	266	266	189	0·068
10.12.29	46	76	30	229	229	153	0·052
							0·069

from Stella's figures on the assumption that the only change involved is the combination, with pre-existing free K-ions, of the bicarbonate ions formed from the carbonic acid; it is quite inconsistent with the hypothesis that the K-ions are new ions drawn from some previously undissociated compound, which would require a value of 0·158. The experiments are difficult ones, and cannot be made very accurate; they involve moreover the assumption that the true metabolic rate of heat production is the same in CO_2 as in N_2; so far, however, as they go they favour the view that an acid artificially introduced into muscle produces a change of osmotic pressure corresponding to the number of its anions set free, drawing upon previously ionised buffers for the cations required. In any case it is clear that when acid ions are set free in muscle artificially the rise of osmotic pressure is not 2·8 times as great as that calculated from their concentration; hence, in the natural accumulation of lactic acid in fatigue, we cannot attribute the excessive osmotic change to a direct action of the acid, but must credit it rather to other causes associated with activity.

V. The Swelling of Muscle as the Result of Exercise.

In a very careful study, 65 years ago, Ranke (1865) showed that in frogs with the circulation intact the water content of the muscles increased, and of the blood decreased, as the consequence of fatigue produced by violent strychnine spasms. Great care was taken in the drying; the material was kept at 100° C. for 48 hours, and then in a vacuum over calcium chloride for another 24. His mean values were as follows:

Fresh muscles: solids, 19·6 p.c.; water, 80·4 p.c.
Fatigued muscles: solids, 17·9 p.c.; water, 82·1 p.c.

Assuming that the total solids of a given muscle were unaltered in amount, and that the change observed was due to absorption of water, we may calculate that as the result of fatigue 100 g. of muscle took up 9·5 g. of water from the blood and lymph.

For blood he found:

Resting animals: solids, 11·7 p.c.; water, 88·3 p.c.
Fatigued animals: solids, 13·0 p.c.; water, 87·0 p.c.

Assuming again that the total solids were unaltered and that the change observed was due to loss of water, we may calculate that 100 g. of blood lost 10 g. of water, as the result of flowing through fatigued muscles.

It is doubtful whether to-day these observations could be bettered, and they allow—with certain approximate assumptions—an interesting calculation to be made of the alteration of osmotic pressure occurring in muscles when exhausted. Let P be the initial osmotic pressure of muscle, ΔP the change produced by fatigue, assuming that no dilution occurs. Actually, however, 100 g. of muscle, containing 77 g. of "free" water,* took up 9·5 g. of water, so reducing its osmotic pressure in the ratio of 77 : 86·5. Hence the final osmotic pressure of the muscle was $77\,(P + \Delta P)/86{\cdot}5$. The blood was originally in equilibrium with the muscle, and had osmotic pressure P. Initially 100 g. of blood contained 88·3 g. of water and (say) 87 g. of "free" water; 10 g. of this was lost to the exhausted muscles; consequently the osmotic pressure rose in the ratio of 87 : 77. Hence the final osmotic pressure of the blood was $87P/77$. But in the end blood and muscle were again in osmotic equilibrium as they were initially, otherwise water would pass one way or the other. Hence $77\,(P + \Delta P)/86{\cdot}5 = 87\,P/77$. Solving this equation we find

$$\Delta P/P = 0{\cdot}27.$$

* See the following paper.

In other words the osmotic pressure of the muscles was increased by 27 p.c. as the result of exhaustion. We realise that the assumptions involved are anything but exact; for example, lactic acid formed in the muscle diffuses into the blood, and not all the water in muscle is confined by semi-permeable membranes. Any errors, however, of this kind tend to make the calculated result too small, so that our conclusion may be restated in the form, *the osmotic pressure of the muscles was increased by more (probably considerably more) than 27 p.c. as the result of exhaustion.* According to Moore (1916) the depression of freezing point of muscles is increased from 0·42° to 0·57° as the result of fatigue, *i.e.*, by 36 p.c. According to the experiments recorded in this paper the depression of vapour pressure of muscles is increased by 50 p.c. as the result of exhaustion. Ranke's muscles were certainly not as fatigued as ours. It is striking how clearly these experiments of Ranke's made 65 years ago showed, almost quantitatively and with the simplest methods, the phenomenon discussed in this paper.

Various recent researches have been devoted to the same phenomenon, the swelling of muscles as the result of exercise. Barcroft and Kato (1915) stimulated the muscles of dogs in the body and found (*a*) an exudation of water from the blood vessels lasting for hours (shown by an excess of hæmoglobin in the venous blood); (*b*) an increase of weight in the stimulated muscles (up to 21 p.c.); and (*c*) a decrease in the specific gravity of the muscles as the result of fatigue. Back, Cogan and Towers (1915) working with frogs with the brain pithed in front of the medulla found that a gastrocnemius, stimulated by induction shocks at 40 per minute for 15 minutes, gained 4·4 p.c. in weight in comparison with the unstimulated control. Such swelling indeed is an inevitable consequence of the rise of osmotic pressure associated with activity. What had not been realised was the magnitude of the osmotic change involved.

Some of the well-known consequences of severe exercise in man, *e.g.*, stiffness and swelling of the muscles, as Barcroft suggested, may be due to the large change of osmotic pressure accompanying fatigue. Such osmotic changes as occur in muscle as the result of severe exercise suddenly undertaken must be rapidly reflected in the blood, and it is hoped to pursue the matter further on this side by observations of the vapour pressure of human blood immediately after, and in recovery from, severe exercise of various kinds.

Summary.

1. The increment previously reported in the rate of heat production of a muscle at rest, following stimulation in nitrogen, is due entirely to a decrease

of vapour pressure resulting from anaerobic activity. It is caused by the slow condensation of water, on its surface, from the Ringer's solution with which the muscle was previously in equilibrium. It disappears completely when the nitrogen is replaced by paraffin oil, in which the transfer of water vapour is impossible. It can be imitated in an unstimulated muscle by washing out the chamber with a more dilute solution, *i.e.*, one of higher vapour pressure, by which a similar transfer of water to the muscle is set up.

2. This change of vapour pressure is directly proportional to the change of osmotic pressure (or of molecular concentration) which causes it. The change of osmotic pressure set up in the fluids of a muscle stimulated anaerobically is nearly proportional to the amount of energy set free ; the number of molecules (or ions) liberated increases rather more than in direct proportion to the energy.

3. The change of osmotic pressure in a muscle stimulated anaerobically can be expressed in absolute units by means of an appropriate calibration. In a frog's sartorius muscle completely fatigued (lactic acid production 0·3 p.c.) the increase of osmotic pressure is the same as would be caused by the addition of 0·35 p.c. of NaCl to Ringer's solution.

4. The increase of osmotic pressure in the fluids of a stimulated muscle is about 2·8 times as great as would be exerted by the lactate ions produced, if dissolved in the " free " water of the muscle. It is 1·8 times as great as would correspond to the lactate ions together with the creatine liberated by the breakdown of phosphagen. It is appreciably greater than can be accounted for by all the chemical changes at present known, or suspected, to occur in stimulated muscle.

5. When an acid (carbonic) is added artificially to muscle, the increase of osmotic pressure corresponds approximately to the anions formed. The cations were free before combination with the acid. Hence, in the natural formation of lactic acid in fatigue, we cannot attribute the excessive osmotic change to a direct action of the acid, but must credit it to other causes associated with activity.

6. The depression of vapour pressure of frog's blood has been measured. Its mean value is that of a 0·725 p.c. solution of NaCl. This, therefore, is the strength of the isotonic solution.

7. The osmotic pressure of frogs' resting muscles, assumed to be isotonic with blood, can be almost exactly accounted for by assuming the known soluble constituents of muscle to be dissolved in its " free " water (see the following paper). There is no latitude for supposing that these constituents are not

dissolved. The osmotic pressure of fatigued muscles cannot be so accounted for. Presumably therefore some hitherto unrecognised substances are liberated as the result of activity.

8. These excessive osmotic changes occurring during activity are the cause of the swelling and may be connected with the stiffness of muscles after severe exercise.

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The State of Water in Muscle and Blood and the Osmotic Behaviour of Muscle.

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I. Introduction.

In the course of the investigation described in the preceding paper by Hill and Kupalov it became necessary to determine the amount of "free" water in muscle, *i.e.*, the weight of water per gramme of muscle which is capable of dissolving in a normal manner (with the normal depression of vapour pressure) substances added to it. For many years, chiefly on the evidence of the experiments of Overton (1902), it has been commonly supposed that a large proportion of the water of muscle exists in some "bound" form, incapable of taking part in the osmotic changes which occur when the tissue is immersed in hypo- or hyper-tonic solutions. From the fact that a muscle swells to much less than twice its initial weight when immersed in a solution of half the initial osmotic pressure Overton concluded "dass nicht das gesammte im Muskel befindliche Wasser in der Form eines Lösungsmittels enthalten sein kann." I have confirmed Overton's experiments (see below) but believe that a very different explanation of them is necessary.

Various other definitions of, and methods of determining, the "free" water of a solution (or a tissue) are possible, and these do not necessarily coincide with each other. Jensen and Fischer (1910) and Jensen (1912) determined the "bound" water by making cooling curves for muscles and calculating the

heat absorbed from the areas of these curves. A comparison of the results with those obtained with solutions of NaCl was believed to allow a calculation of the "bound" water. In fresh muscle they found 4 p.c. of the total water to be "bound," in muscle killed by freezing and thawing 14 to 17 p.c., in muscle heated to 100° C. 22 p.c.

A very obvious objection was raised by Rubner (1922), viz., that thermal conduction may be quite different in muscle and in solutions of NaCl. Rubner proposed an alternative method based on the same general idea, viz., that "bound" water may be defined as that which cannot be frozen out by cooling the tissue to such temperatures as — 20° C. The material to be investigated was cooled to a low temperature for two hours and then dropped into a water calorimeter, the heat required to melt it being measured. He found that 1 gramme of dry substance was associated with the following amounts of "bound" water :—

Egg-white	0·33 g.
Blood corpuscles	0·63 g.
Elastic tissue	0·44 g.
Blood vessels	0·45 g.
Beef muscle (dead)	0·76 g.
Beef heart-muscle (dead)	0·64 g.
Frog's muscle (alive)	0·90 g.

The frog's muscle was cooled to — 18° C. and 100 g. of muscle contained 61·7 g. of "free" water (mean of 11 observations); similar muscle contained 79·8 p.c. of total water.

Rubner's method also is not free from possible objection :—

(i) It assumes that the "bound" water is the same at — 20° C. as at the ordinary temperatures in which we are interested; the "binding" of water by a hydrophilic colloid is likely to be an exothermic reaction, in which case it might proceed appreciably further at a low temperature than at a high.

(ii) It assumes that no reactions other than the melting of ice occur when the temperature rises.

(iii) The specific heats of the solid constituents of muscle may not be very accurately known, or the heats of their solution negligible. It is possible, moreover, that water prevented from freezing by association with hydrophilic colloids may nevertheless be capable of dissolving substances present in the tissue.

Rubner's method was employed by Thoenes (1925). The latter found in a gelatin jelly about 2 g. of water " bound " by each 1 g. of dry material; in agar jelly about 4 g. In the muscles of young animals he found about 2 g. of " bound " water per 1 g. of dry substance, in those of old animals about 1 g. The muscles were frozen and thawed, and after passing into rigor were frozen again and the heat of thawing measured. " Auf diesem Wege," he claimed, " gelingt der Nachweis einer Änderung der Wasserbindung in Zustande der Starre mit grösserer Sicherheit."

Robinson (1928) applied the same method to investigating the hardiness of insects exposed to low temperatures during winter. In some insects as much as one-half of the water they contain may be " bound," in the sense that it is not frozen by cooling to $-20°$ C. In hardy insects (Promothea) exposed to low temperatures the proportion of water " bound " may increase from about 8 p.c. at the start to over 40 p.c. after two or three weeks' exposure. The method employed by Thoenes and by Robinson is discussed in detail by Gortner (1929).

Another definition of, and another method of determining, the " free " water were suggested by Newton and Gortner (1922), who added a known amount of cane sugar to expressed plant juice and measured the resulting depression of freezing point. By comparing this with the depression caused by adding the same amount of sugar to an amount of water equal to the *total* quantity contained in the juice, they were able to show that an appreciable fraction of the water was " bound," in the sense that it took no part in the solution of the cane sugar. In one case in which the solids made up 0·178 g. per 1 g. of juice, of the total water (0·822 g.) 0·130 g. was found to be " bound." Newton (referred to by Gortner (1929)) has employed the same method of studying the state of water in the sap of winter wheat, in drought-resistant crops, and in the press juice of grasses. The method is of general application, and similar in principle to the one employed in the present investigation. It requires, however, greater quantities of fluid, and for accuracy apparently greater concentrations of solute, and it is open to the possible objection that at ordinary temperatures the amount of water " bound " may be less than at the freezing point. A similar method was used by Straub (1927) in his investigations of milk.

Another method depends upon the fact that a soluble substance which is capable of penetrating the cells of a tissue will finally attain equality of concentration in the water of an external solution and in the " free " water of the tissue. A quantitative determination of the amount of the substance in

question present in 1 g. of the tissue, when in diffusion equilibrium with a given external concentration, allows an estimate to be made of the "free" water. This method, suggested by P. Eggleton and H. V. Horton, was applied by them to the case of urea distributing itself between Ringer's solution and muscle; their results they have kindly allowed me to report here. Not many substances, however, if any, are really suitable for the purpose: (*a*) They must be capable of penetrating the tissue rapidly and completely; (*b*) they must not be dissolved in, or adsorbed by the solid material of the tissue—(*a*) and (*b*) are an unusual combination of properties; (*c*) they must be in the same physical state in the solution as in the tissue—CO_2 is inadmissible because it produces HCO_3 ions in a buffered medium; (*d*) they must be susceptible of accurate quantitative estimation; (*e*) they must be non-electrolytes, otherwise the assumed equality of concentration will be prevented by the Donnan membrane effect.

An analogous method, however, can be employed if pieces of tissue are available which are small enough to allow *water* to redistribute itself in a reasonable time between them and a small quantity of solution in which they are immersed. Imagine, for example, that four small muscles are soaked for some time in normal oxygenated Ringer's solution (R). Let them be carefully blotted and weighed, and let their weight be (say) 1 g. Let them be mixed with a small weighed quantity of a solution, containing the constituents of Ringer's fluid in twice the normal concentration (2R); let the amount of water in this 2R solution be 0·8 g. Let the muscles and the solution be stirred together for (say) 16 hours in a stoppered tube containing oxygen; in that time the osmotic pressures of muscles and solution will have been equalised by the diffusion of salt inwards and of water outwards. Let the depression of vapour pressure of the solution be equal finally (say) to that of a solution 1·5R. By water passing out and by salts passing in, therefore, the osmotic pressure of the muscle has risen 50 p.c., while that of the solution has fallen 50 p.c. This can only be the case if the "free" water of the original muscle was the same in amount as the water of the original solution; hence the "free" water of the muscle in this case was 0·8 g.

We have taken a simple case for purposes of illustration, but the method is general and requires no substance of peculiar properties as its basis. The only special requirement is an accurate means of measuring the depression of vapour pressure of a small amount of solution. This is available in the thermal method recently described (Hill, 1930) and referred to in the preceding paper

The practicability of the method as applied to muscles was not realised at

first; blood, therefore, and solutions of casein and egg-white, were first studied, and their " free " water determined, in the hope that the results might throw light upon the analogous problem of muscle. The method used was to add various soluble substances to the solution in question and to compare the depression of vapour pressure caused thereby with that due to adding the same amount of the substance to a known amount of salt solution. For example, let us add 1 g. of cane sugar to 10 g. of blood and measure the difference of vapour pressure between the resulting solution and the original blood; let this difference be 125 (arbitrary) units. Let us now add 1 g. of cane sugar to 10·1 g. of 1 p.c. NaCl solution (containing 10 g. of water) and measure the difference of vapour pressure between the resulting solution and the original 1 p.c. NaCl; let this difference be 100 units. It is clear then that the " free " water in the 10 g. of blood is 100/125 of that in the 10·1 g. of NaCl solution; it is 8 g. Various substances, and in various concentrations, have been added to the solutions in question, and the results are described in subsequent sections.

II. The State of Water in Blood.

Mammalian blood contains about the same relative amount of water as amphibian muscle, viz., 80 g. of H_2O per 100 g. of blood. Its chief protein, moreover, hæmoglobin, is an efficient buffer, being the ionised alkali salt of a weak acid, the undissociated protein being formed when a stronger acid is added :—

$$K^+ + Hb^- + H^+ + A^- \rightarrow K^+ + A^- + HHb.$$

In both respects blood, regarded as a physico-chemical system, is similar to muscle. Its hydrogen ion concentration is about the same. Very precise data are available as to the chemical constitution of normal mammalian blood; its freezing point (— 0·56 to — 0·57° C.) is accurately known, and experience has demonstrated that " mammalian Ringer-Locke's solution " of the same freezing point is isotonic, at any rate approximately, with the tissues which were previously in equilibrium with the blood. It is instructive, therefore, to calculate the sum of the molal concentrations (gramme-molecules per 1000 g. of *water*) of the constituents of blood, and to compare this sum with the molal concentration of a NaCl solution which is known to be isotonic with the blood.

Abderhalden's (1898) analyses of the blood of two cattle, two sheep, two horses, two dogs, one goat, one pig, one rabbit and one cat, allow the following mean values to be calculated. It is realised that by taking the mean of his 12 values for each constituent we obtain a result which is true in detail for no

particular animal; since, however, our only object is to find the *sum* of the molal concentrations for mammalian blood, no error is introduced by taking the mean, and the result is more accurate.

Table I.—Mean Values calculated from Abderhalden's Data for Mammalian Blood.

Substance.	g. to 100 g. blood.	g. to 100 g. H_2O.	Molal concentration.	Remarks.
H_2O	80·2	—	—	—
Hb	12·5	15·6	0·0023	Molecular weight assumed 67000 (Adair (1925), (1928)).
Sugar	0·0790	0·0980	0·0054	—
Na	0·2460	0·3060	0·1331	—
K	0·0790	0·0980	0·0252	—
Ca	0·0044	0·0055	0·0010	Assumed 70 p.c. as free Ca ions.
Mg	0·0031	0·0039	0·0016	—
Cl	0·2890	0·3604	0·1015	—
Total P	0·0314	0·0392	0·0088	See note.
		Sum	0·2789	

Note.—The molal concentration for total P assumes that the number of dissolved molecules containing phosphorus is 70 p.c. of the number of phosphorus atoms. According to private information from Mr. P. Eggleton the phosphorus distribution in blood is approximately as follows, expressed as mg. P. per 100 g. blood. Inorganic phosphate, 4½; pyrophosphate, 2½; adenylic acid, 12; hexosemonophosphate, 1½; hexose diphosphate, 4; the remaining phosphorus being present in lipins, probably not dissolved.

Abderhalden's list, however, although it makes up nearly 87 p.c. of the sum of the molal concentrations, must be supplemented by the following, data for which have been obtained, in consultation with Mr. P. Eggleton, from various sources.

Table II.—Constituents of Mammalian Blood.

Substance.	Concentration assumed.	Molal concentration.	Remarks.
Protein other than Hb	One-third of the value for Hb	0·0008	A rough estimate, but the value is practically negligible in any case.
Bicarbonate	50 c.c. combined CO_2 per 100 g. blood	0·0280	—
Lactate	16 mg. per 100 g. blood	0·0022	—
SO_4	....	0·0003	—
Urea	0·030 p.c. in blood	0·0062	—
Amino acids	0·006 p.c. N in blood	0·0053	One N atom assumed to each molecule.
Creatine	0·007 p.c. in blood	0·0006	—
Creatinine	0·001 p.c. in blood	0·0001	—
Uric acid	0·002 p.c. in blood	0·0002	—
	Sum	0·0437	

The sum, therefore, of the molal concentrations of all constituents, obtained by adding the results of Tables I and II, is 0·3226. This is equal to the sum of the molal concentrations of the ions of a 0·1613 molal NaCl solution, which is 0·943 g.* in 100 g. H_2O. Mammalian Ringer-Locke's solution, as given by Bayliss (1924, p. 211), is isotonic with 0·971 g. NaCl in 100 g. H_2O, while the freezing point of human blood corresponds to 0·96 g. NaCl in 100 g. H_2O. If we assume that about 2 p.c. of the water of blood is bound by the protein, the remaining 98 p.c. being free to dissolve the constituents of blood in a normal manner, then the sum of the molal concentrations of the constituents of blood agrees exactly with the observed freezing point, and very closely with the usual constitution of mammalian Ringer-Locke's solution. It seems probable (i) that the water of blood is almost entirely "free," in the sense of being able to dissolve chemical substances in a normal manner, and (ii) that the constituents of blood are to be regarded as normally dissolved, and as exerting their normal osmotic pressures, in the water of the blood.

This conclusion has been tested experimentally by finding the depression of vapour pressure caused by adding various substances to blood, and comparing this with the depression of vapour pressure caused by adding the same substances in similar amounts to a 1 p.c. NaCl solution. The depression of vapour pressure has been reckoned in both cases *per gramme of substance added to 100 grammes of water*, the water content of the blood being measured by drying to a constant weight on a water bath at 100° C. Results have been expressed in terms of the ratio :

$$\frac{\text{(Vapour pressure depression caused by adding 1 g. of solute to 100 g. of water in 1 p.c. NaCl solution)}}{\text{(Vapour pressure depression caused by adding 1 g. of solute to 100 g. of water in blood).}}$$

If the ratio be unity we conclude that the whole of the water of blood is free to dissolve in a normal manner chemical substances added to it. If the ratio be less than unity we must conclude that some of the water of blood is bound by the colloidal or other bodies there present, and so is unable to assist in the solution. If it be greater than unity we must assume that the substance added is somehow removed from free solution by the presence of other bodies, *e.g.*, by surface adsorption, or by "solution" in or combination with the protein or lipins.

* [*Added in proof.*—This is almost precisely the mean value for the NaCl solution which has the same vapour pressure as human blood exposed to 5 per cent. CO_2, as found by Margaria in recent experiments here.]

A further test can be applied by adding to blood, not chemical bodies as described above but water. If we regard as fixed the total number of ions or molecules present in a given amount of blood, and add water to it, the depression of vapour pressure observed at any stage should be inversely proportional to the total volume of "free" water present at that stage. It is found by experiment that the reciprocal of the difference of vapour pressure between water and a mixture of blood and water is a linear function of the amount of water added to a given amount of blood. This is an expression of the relation, true for dilute solutions, $PV = $ a constant, P being the total osmotic pressure of all the dissolved constituents and V the volume of water in the solution. By plotting the relation between $1/P$ and V and extrapolating it backwards to the axis of V it is possible to determine the amount of "free" water in the original blood. The results so obtained agree with those found by the previous method.

The depression of the vapour pressure of a solution below that of pure water, or the difference of vapour pressure between two solutions, was measured by the thermal method described recently (Hill, 1930). Two similar pieces of filter paper dipped in the two fluids and allowed for a few moments to drain are placed on the two opposite faces of a symmetrical thermopile. The instrument is mounted in a moist chamber and placed in a thermostat. The temperature of the present observations was 20·25° C. The difference between the rates of evaporation of the two fluids is proportional to the difference between their vapour pressures; the former causes a difference of temperature between the two faces, which becomes steady in 30 to 45 minutes; this may be used as a measure of the latter. Thus the difference of vapour pressure required is directly proportional to the electromotive force developed in the thermopile, as read by a potentiometer and a sensitive galvanometer, and can be expressed in absolute units by calibrating the apparatus with solutions of known vapour pressure, *e.g.*, of cane sugar, NaCl or KCl. With the instrument hitherto employed,* if the difference to be observed was not too small, the average error of a single reading was about 1½ p.c. The sensitivity was such that the difference of vapour pressure between water and 1 p.c. NaCl solution gave a deflection of about 1200 mm. on the galvanometer scale.

Defibrinated slaughter-house blood was used. In some cases this was centrifuged and the corpuscles alone employed. It appeared to make no

* [*Added in proof.*—In Dr. Margaria's hands the same instrument, and other similar ones, employed with human blood are considerably more accurate than originally claimed.]

difference whether the blood was laked or not. If cane sugar, for example, be added to intact blood, water is immediately withdrawn from the corpuscles in amount sufficient to make the osmotic pressure of the external fluid equal to that of the internal; the rise of osmotic pressure is the same as if the corpuscular envelope were absent. This would not be the case were the osmotic pressure normally existing inside the red cells different from that of the serum. The absence of such a difference was demonstrated as follows.

A mass of centrifuged corpuscles was carefully oxygenated and separated into two parts, each being placed in a stoppered bottle. One part was laked, without exposure, by freezing to about − 15° C. and thawing. The difference of vapour pressure between the two fluids, laked and unlaked, was then measured and found to be negligible. In this respect, at any rate, a true equilibrium appears to exist across the corpuscular membrane.

Various substances have been added to blood, or to blood corpuscles:— $NaCl$, KCl, $CaCl_2$, cane sugar, urea, creatine, succinic acid, lactic acid, and water. In the case of the acids, in order to avoid if possible any error due to driving out carbon dioxide, the blood was shaken previously at 38° C. for half an hour, with continual changes of air. The lactic acid was prepared for me free from lactide by Mr. P. Eggleton.

In addition to the experiments given in Table III below, three series of experiments were performed in which water was added to blood corpuscles in varying amounts, and the free water content computed from the relation between observed vapour pressure depression and amount of water added. In these three series the ratio (free water)/(total water) had the following values: 0·93, 0·92, 1·00; mean 0·95.

We see that for $NaCl$, KCl and cane sugar, substances which are incapable of penetrating living tissues in their normal state, the ratio is about unity, having a mean value of 0·97. The addition of water led to almost exactly the same number, viz., 0·95. For $CaCl_2$ in a single experiment the value was rather greater, 1·06, but we should expect a certain amount of the calcium to be precipitated. Urea and creatine gave a greater number, the mean value being 1·17. It will be seen later that in casein solutions and in egg white the ratio for urea is not far from unity. In blood, therefore, the lower value is probably due to the removal of part of the urea from solution, perhaps by adsorption to, or combination with, the colloidal material or the lipins present; urea is singularly capable of penetrating living cells, which is probably a sign of its readiness to be combined or adsorbed. Succinic acid and lactic acid

Table III.

The ratio given is B/A where A = the depression of vapour pressure caused by adding 1 g. of the substance in question to 100 g. of total water in blood, and B = the depression of vapour pressure caused by adding the same quantity of the substance to 100 g. of water in a NaCl solution containing 1 g. of NaCl in 100 g. of water. For a substance which is not adsorbed B/A is a measure of the ratio, "free" water/total water. The total water in the blood was determined by drying at 100° C. on a water bath.

Fluid.	p.c. H_2O.	Substance added.	g. to 100 g.	g. to 100 g. H_2O.	Ratio B/A.
Blood	78·5	NaCl	1·03	1·313	0·99
Blood	82·5	NaCl	0·25	0·303	0·95
Corpuscles	70·2	NaCl	0·283	0·404	0·955
Blood	82·5	KCl	0·40	0·485	0·93
Corpuscles	71·1	KCl	4·50	6·33	0·97
Blood	78·5	Cane sugar	10·70	13·63	1·02
Blood	82·5	Cane sugar	2·50	3·03	1·02
Corpuscles	70·2	Cane sugar	2·50	3·57	0·94
Blood	82·5	$CaCl_2$	0·40	0·485	1·06
Blood	78·5	Urea	2·00	2·55	1·13
Blood	78·5	Urea	0·50	0·637	1·24
Corpuscles	71·0	Urea	0·513	0·723	1·11
Corpuscles	71·0	Urea	0·355	0·500	1·18
Corpuscles	71·1	Urea	7·138	10·035	1·09
Corpuscles	70·1	Creatine	0·80	1·140	1·25
Blood	78·5	Succinic acid	0·98	1·248	1·70
Blood	78·5	Succinic acid	0·49	0·624	2·08
Blood	78·5	Lactic acid	0·455	0·580	1·34
Blood	78·5	Lactic acid	0·321	0·410	1·24
Blood	78·5	Lactic acid	0·317	0·403	1·24
Blood	78·5	Lactic acid	0·228	0·289	1·39
Corpuscles	71·8	Lactic acid	0·201	0·280	1·32
Corpuscles	71·8	Lactic acid	0·248	0·345	1·35

also gave a greater number; for the latter the mean value was 1·31. This low value is partly due to two factors :—

(*a*) In the solution of the acid in unbuffered NaCl solution a certain small degree of ionisation occurs; the effect of the H-ions is added to that of the lactate (or succinate) ions, consequently the numerator of the ratio is rather too large. In the solution of the acid in blood, however, the powerful buffering prevents any appreciable quantity of hydrogen ions from appearing. The total effect, however, is not great. To allow for it might decrease the ratio to 1·25.

(*b*) A small amount of bicarbonate remaining in the blood may have been broken up by the acid, and CO_2 driven off. For such lactate ions as simply replaced bicarbonate ions no vapour pressure change at all would be registered; consequently the ratio would be lowered. No

reasonable estimate, however, of the CO_2 driven off will account for all the effect observed ; it is more likely that a certain amount of the lactic or succinic acid is adsorbed, or removed from solution, in the same way as urea and creatine appear to be.

On the evidence, therefore, of the results obtained by adding NaCl, KCl, cane sugar and water, we may conclude that nearly the whole of the water of blood, say, 97 p.c. of it, is free to exert its normal behaviour as a solvent. This agrees with the deduction made above from the analyses and the observed freezing point. Some substances, however, such as urea and creatine, and probably lactic and succinic acids, fail to exert their full effect on the vapour pressure owing to a slight degree of adsorption on, or combination with, the colloidal constituents or the lipins of the blood. It would, of course, be possible to argue that some of the NaCl, KCl, or cane sugar also is adsorbed, the effect being exactly compensated by the demobilisation (*qua* solvent) of part of the water. It is not easy, however, in that case to explain the fact that the addition of water leads to the same value of the ratio as the addition of any of these three bodies. It is much simpler to suppose that nearly all the water is free and that these substances are normally dissolved in it. This view is confirmed by the experiments on casein solutions, on egg yolk and on egg white to be described next.

III. The State of Water in Protein Solutions.

The method described above for the case of blood was applied with little change to (*a*) a strong solution of casein in N/10 NaOH, (*b*) egg-white concentrated by evaporation, and (*c*) egg-yolk. The total water content was determined by drying at 100° C. on a water bath and finally at 110° C. in an oven. The stronger casein solutions were very viscous. The strips of filter paper, therefore, were left in them for some time to become properly moistened. They were then removed and laid on a glass plate, and the excess of solution was rapidly cleaned off with a pair of forceps and a wire. In spite of the difficulty in manipulation, apparently very reliable results were obtained. The egg-white was left in a vacuum desiccator for three days over calcium chloride. In that time half the water evaporated and the depression of vapour pressure was doubled.

From Table IV we see that for casein solutions and egg-white the mean value of the ratio "free" water/total water for *all* substances added is 0·98. Apparently, therefore, at room temperature only about 2 p.c. of the water is

Table IV.

Change of vapour pressure due to adding various substances to casein solution, egg white and egg yolk. The ratio given is B/A, where A = the depression of vapour pressure caused by adding 1 g. of the substance in question to 100 g. of water in the solution investigated, and B = the depression of vapour pressure caused by adding the same quantity of the substance to 100 g. of water in a 1 p.c. NaCl solution. For a substance which is not adsorbed B/A is a measure of the ratio, "free" water/total water.

Fluid.	p.c. H_2O.	Substance added.	g. to 100 g.	g. to 100 g. H_2O.	Ratio B/A.
Casein solution	85·2	NaCl	0·803	0·942	0·94
Casein solution	85·2	Urea	1·946	2·284	1·00
Casein solution	85·2	KCl	1·034	1·213	0·97
Casein solution	85·2	Cane sugar	8·22	9·65	0·97
Casein solution	83·1	NaCl	0·826	0·994	1·00
Casein solution	83·1	Urea	1·616	1·945	1·06
Casein solution	83·1	KCl	0·889	1·070	0·92
Casein solution	81·3	NaCl	2·84	3·49	0·99
Casein solution	80·56	NaCl	1·95	2·424	0·98
Egg white	78·7	KCl	2·12	2·70	0·98
Egg white	78·7	Cane sugar	1·412	17·95	0·94
Egg white	78·7	Urea	2·83	3·60	1·01
Egg yolk	47·0	KCl	1·189	2·53	0·85

"bound." In the single experiment on egg yolk there was about 15 p.c. of water "bound" by 53 p.c. of solid. The two protein solutions, therefore, give practically the same result as blood, in which we concluded that only about 3 p.c. of the water is "bound" by the solid constituents.

IV. The State of Water in Muscle.

(*a*) *The Solution of Urea in Muscle.*—The following experiments were suggested and carried out by Mr. P. Eggleton and Mr. H. V. Horton, who have kindly allowed me to report the results. One, two, or three sartorius muscles, from large Hungarian frogs, were soaked for about 4 hours in a small quantity of Ringer's solution containing initially 0·14 p.c. urea. Each sartorius weighed about 250 mg. and the weight of solution employed was about twice that of the muscles. The opposite muscles from the same animals were similarly soaked in Ringer's solution without urea. Urea was chosen (*a*) owing to the extreme readiness with which it penetrates the tissues; a comparison of the results obtained after 3 and 5 hours' soaking showed that diffusion was complete in the shorter time, so 4 hours' soaking was taken as standard; (*b*) because it is not known to react in any way with the constituents of muscle; and (*c*) because the amount of it in the solution can be measured with considerable

accuracy as total nitrogen. The muscles were weighed before and after soaking; no important change of weight occurred. A measurement of the total nitrogen was made four times in every experiment, once each on the two solutions before soaking, once each after soaking. From the results the quantity of urea which had disappeared from the solution was calculated; assuming that it was dissolved in the "free" water of the muscle, attaining there a concentration equal to that in the fluid outside, the quantity of "free" water in the muscle was computed and expressed as a fraction of the weight of the muscle. The results are given in Table V.

Table V.

"Free" water of frog's muscle, calculated from the distribution of urea between muscle and external solution, and expressed as a percentage of the weight of the muscle. The number in brackets after each result is a weighting factor, depending partly upon the quantity of material used, partly upon an estimate by the observers of the general reliability of the experiment.

74 (1); 75½ (1); 67 (1); 91 (1); 90 (1); 83½ (2); 79 (3).

Weighted mean = 80.

Accepting the assumptions made in the calculation the mean value corresponds to a quantity of "free" water equal to 80 p.c. of the weight of the muscle, equal, that is to say, to the total water; apparently none of the water of muscle is "bound" by the solids. It is true that the same result might have been found if part of the water were "bound," and a corresponding part of the urea adsorbed. If urea be added to blood a small fraction of it—as shown, above—does not go into solution in the water of blood; in egg-white and casein solutions, however, the whole of the added urea is apparently simply dissolved. The urea experiments, therefore, cannot be regarded as a final proof that all, or nearly all, the water of muscle is "free"; they have been confirmed, however, by those to be described in the next section, which involved a different procedure and different substances.

(*b*) *The "Free" Water of Muscle determined by Vapour Pressure Measurements.*—Two methods have been employed to determine the "free" water of muscle, the "single" and the "differential." Both are applicable to the case of a resting muscle; for muscles fatigued or in rigor, however, the latter only can be used.

In the "single" method several small muscles (preferably the gastrocnemii and the vasti interni (Marshall) of one or two small frogs, which can be prepared practically without injury) were first soaked for several hours in normal

oxygenated Ringer's solution (R), isotonic—see the preceding paper by Hill and Kupalov—with 0·703 p.c. NaCl. Since, apart from CO_2, the muscles were already practically isotonic with this solution, 5 or 6 hours of soaking should bring them fairly accurately into equilibrium with it. They were then withdrawn one by one from the solution, freed from all adherent fluid by rapidly blotting with filter paper, and dropped into a weighed glass tube of capacity about 10 c.c. When the muscles were all in the tube the whole was weighed, and so the weight of the muscles obtained to the nearest 1 mg.

To the muscles in the tube was now added an accurately weighed amount of Ringer's solution of twice the normal strength (2R), which is isotonic with 1·406 p.c. NaCl. It can be shown mathematically that the method is most sensitive and accurate when the quantity of 2R-solution added contains an amount of water equal to the amount of " free " water in the muscles. Since the latter is about 80 p.c. of their weight it has proved convenient in practice to add a quantity of 2R-solution containing an amount of water precisely equal to 80 p.c. of the weight of the muscles. The tube was then filled with oxygen and stoppered, and the muscles and the 2R-solution were left 15 to 20 hours, so as to come into diffusion equilibrium with one another; during that time the tube was kept slowly revolving so as to ensure adequate mixing. The final result was that muscles and solution attained an osmotic pressure about equal to that of 1·5R. The muscles weighed finally 10 to 20 p.c. less than they did at the start, but were usually in excellent condition and very excitable.

It is essential that a sufficient period should be allowed for equilibrium to be attained; see the last section of this paper; with muscles of 0·2 to 0·3 g. it is best to leave the tube rotating during the night and to complete the observations next day. If sufficient time be not allowed the osmotic withdrawal of water from the muscles will not be complete, the osmotic pressure of the solution will be too high, and the " free " water of the muscle calculated from it too low. It is *not* advisable to use muscles weighing more than 0·3 g., otherwise the time required for osmotic equilibrium to be attained (which for muscles of similar shape varies as the 2/3 power of the weight) will be so great that the muscles may have depreciated; moreover there is the difficulty of an adequate supply of oxygen to the interior (Hill, 1928) if the muscles be too large. The process of equilibration consists partly of a diffusion of water from the muscle into the solution outside, and partly (so far as semi-permeable membranes allow) of a diffusion of salt from the solution into the muscle. It is impossible to imagine a difference of osmotic pressure to exist between two different parts

of the system once equilibrium has been attained, for the membranes involved are far too thin to be able to stand any considerable mechanical stress.

After 15 to 20 hours mixing the tube was opened, the muscles removed, and the difference of vapour pressure measured between the fluid left in the tube and the original Ringer's solution (R). Two or three readings of this vapour pressure difference were generally made, and on the same day the apparatus was calibrated with the solutions R and 2R on its two faces. To take an example, 2 g. of muscle after equilibration with R was mixed with an amount of 2R containing 1·6 g. of water. After 18 hours mixing the final vapour pressure difference between the fluid in the tube and the solution R was 127 (arbitrary units). On the same day the vapour pressure difference between R and 2R gave 249 units. Thus the final osmotic pressure of the mixture in the tube was the same as that of a solution $(1 + \frac{127}{249})$ R $= 1 \cdot 51$ R.

We may argue as follows, if x be the amount of "free" water per 1 g. of muscle :—

$$(x \times \text{weight of muscles})\,\text{R} + (\text{weight of water in 2R added})\,2\text{R}$$
$$= (x \times \text{weight of muscles} + \text{weight of water in 2R added})\,1 \cdot 51\text{R}.$$

This merely expresses the fact that when equilibrium has been attained the dissolved substances originally present (*a*) in the muscle at osmotic pressure R, and (*b*) in the fluid added at osmotic pressure 2R, have been redistributed to give a uniform osmotic pressure (observed) 1·51R. Hence

$$x = \frac{1 - 0 \cdot 51}{0 \cdot 51} \times \frac{\text{weight of water in 2R added}}{\text{weight of muscles}}$$
$$= 0 \cdot 49 \times 0 \cdot 8/0 \cdot 51 = 0 \cdot 77.$$

Therefore 1 g. of muscle contained 0·77 g. of "free" water.

The first six experiments of Table VI were made by this method. It is valid so long as the condition of the muscle may be assumed to remain constant during equilibration with 2R. It cannot be applied when the initial osmotic pressure is not accurately known (as in fatigue) or when the osmotic pressure alters of itself during equilibration (as when anaerobic conditions are necessary, in order to prevent recovery). In such cases the "differential" method must be employed, in which an unknown initial osmotic pressure or progressive osmotic changes during equilibration are automatically allowed for.

In the "differential" method the muscles were divided into two lots, those from the right leg of an animal being allotted to one, those from the left leg to the other. The procedure was much as before, except that twice as many

frogs and two tubes—instead of one—were used. In one tube (A) was placed one lot of muscles together with a weighed quantity of Ringer's solution (R), containing an amount of water equal to 0·8 of the weight of the muscles. In the other tube (B) was placed the other lot of muscles, together with a weighed quantity of 2R-solution containing the same relative quantity of water. After prolonged equilibration the vapour pressure difference was measured between the fluids in tubes A and B, and expressed as a fraction f of that between R and 2R. As before, if x be the quantity of "free" water in 1 g. of the original muscles, it can be shown that

$$x = \frac{1-f}{f} \times \frac{\text{weight of water in 2R added}}{\text{weight of muscles}}.$$

There are several advantages in the "differential" method :—

(*a*) A preliminary soaking in R-solution is unnecessary ; the initial osmotic pressure of the muscles need not be known, all that is necessary is that it should be the same in both lots.

(*b*) Changes occurring in the muscles during the prolonged second equilibration, which would be fatal in the first method, balance out exactly in the second, since they affect the contents of tubes (A) and (B) alike.

(*c*) If the muscles be first equilibrated with R-solution, and if a further observation be made of the difference of vapour pressure between R and the contents of tube (A), we can calculate not only the "free" water of the muscles but the increase of their osmotic pressure, resulting (say) from fatigue or heat rigor. If, for example, the final difference of vapour pressure between R and the contents of tube (A), expressed as a fraction of that between R and 2R, be f', the rise of osmotic pressure in the contents of tube (A) must be equal to that of a solution f'R. Let us suppose the osmotic change in question to have occurred in the muscle alone, and not to have been shared with the fluid around it ; then the rise of osmotic pressure would have been greater in the ratio

$$\frac{\text{total "free" water in tube}}{\text{"free" water in muscle}},$$

which can be shown to lead to the simple expression,

Rise of osmotic pressure in muscle = that of a solution $f'\mathrm{R}/(1-f)$.

In a few experiments, instead of using 2R as the test solution, a solution of urea in Ringer's fluid was employed. This gave very consistent and accurate results, the muscles being in excellent condition after prolonged soaking even

in fairly strong solutions. The value, however, of the "free" water found by the use of urea tended to be rather higher than by that of the solution 2R, and it was recalled that in blood—see Table III above—values for the "free" water obtained with urea were slightly, but definitely, too high. Urea is a peculiar substance, its extreme ability to penetrate living cells may well imply a high solubility in the lipins of the tissue, or a great liability to adsorption: and if some of the urea in the test fluid were removed from free solution by adsorption or otherwise it would appear as if the "free" water of the tissue were greater than it really is. Hence, although the results were in good agreement with those of Eggleton and Horton described above, and although the method employing urea seemed to work so well, it was thought wiser to avoid its use for fear of introducing a small constant error. There is little danger of this with the 2R-solution, the chief constituent of which is NaCl, a substance very unlikely not to remain in free solution.

In Table VI are given the results for resting muscles, every experiment made

Table VI. Experiments to determine the "free" water of resting muscles, made with Ringer's solution of twice normal strength (2R).

D = "differential" method; S = "single" method; v = very; g = good; p = poor.

The weighting factor is adjusted to take account (*a*) of the final state of the muscles as determined by stimulation, and (*b*) of the general reliability and type of the experiment. The equilibration was at room temperature (about 16° C.).

Three experiments with urea instead of 2R are included at the end of the table, but no account is taken of them in the mean value.

Date, 1930.	Average weight of muscles, grammes.	Period of equilibration, hours.		Type of Experiment.	Final condition.	Weighting factor.	Free water fraction.
		Preliminary in R.	In 2R.				
19–20 Feb.	0·27	6·9	15½	S	v.g.	3	0·73
19–20 ,,	0·23	6·5	16	S	v.g.	3	0·73
21–22 ,,	0·22	5	16½	S	v.g.	3	0·77
21–22 ,,	0·27	5	17	S	g.	2	0·79
21–22 ,,	0·19	5	17½	S	g.	2	0·82
21–22 ,,	0·19	5	18	S	v.g.	4	0·80
5–6 Mar.	0·29	2	17	D	v.p.	1	0·93
5–6 ,, ...	0·26	1	19½	D	p.	2	0·82
21–22 Mar.	0·29	6¾	18	D	v.g.	6	0·76
21–22 ,,	0·30	6¾	19½	D	v.g.	6	0·735
6–7 Mar.	0·34	1	(urea) 20½	D	v.g.	—	0·83
17–18 Mar.	0·25	3	(urea) 17	D	v.g.	—	0·83
17–18 ,,	0·45	2½	(urea) 19½	D	v.g.	—	0·80

The mean value of the "free" water fraction, taking due account of the weighting factors, is 0·77.

being recorded. Some of the observations were, on technical grounds, *e.g.*, state of muscles at end, consistency of vapour pressure readings, type of experiment (" differential " being preferable to " single "), etc.—more reliable than others, and the relative reliability of the result was assessed by the observer and expressed by a weighting factor given in the penultimate column. The mean value of the " free " water fraction, taking due account of these weighting factors, is 0·77. It is possible that the true value is slightly greater than this: any error due to incomplete equilibration would make the result too low, and it is not certain that equilibration is quite complete even in 18 hours. The true value cannot, however, be higher than 0·80 or 0·81, which is that of the total water fraction. There is obviously very little water " bound " in the resting muscle. So far as they go the urea experiments gave a slightly higher value than those made with 2R; it may be that they give the truer value, owing to the rapid penetration of urea; for reasons, however, which we have discussed above no account was taken of them in calculating the mean.

In Table VII are given results for fatigued muscles, and for muscles in heat rigor. The " differential " method alone was used, and a weighting factor was allotted, as described above.

The experiments on heat rigor were the simpler. The procedure was identical with that for resting muscles, except that :—

(*a*) The solutions R and 2R contained M/50 or M/100 NaCN to prevent oxidation and partial recovery.

(*b*) The tubes were filled with nitrogen instead of oxygen for the same reason.

(*c*) When all was complete, and the tubes filled and stoppered, they were immersed for half-an-hour in water at about 45° C., so that the muscles inside then went into heat rigor.

After prolonged equilibration the " free " water was determined as before, and in addition, in some experiments, the osmotic change due to rigor was measured by comparing the vapour pressure of the final fluid in the R-tube with that of the original R-solution (for details see above). The result was expressed in the following way :—The osmotic change in the fluid in the R-tube was measured; this is entirely due to the muscles; assume the whole change to be concentrated in the muscles, and express it in terms of p.c. NaCl added to R-solution. To say that the muscles, originally isotonic with 0·703 p.c. NaCl, have undergone an osmotic change equivalent to 0·455 p.c. NaCl, implies that if all the products of rigor had been kept inside the tissue and not

Table VII.—Experiments to determine the "free" water of fatigued muscles, or of muscles in heat rigor, made with Ringer's solution of twice normal strength (2R).

All experiments by "differential" method. The weighting factor is adjusted to express the general reliability of the experiment. The osmotic change is expressed in terms of NaCl as explained in the text. The lactic acid produced is calculated as a fraction of the original free water of the muscles (assumed to be 77 p.c. of their weight). Two experiments with urea instead of 2R are included at the end of the table, but no account is taken of them in the mean value.

Ri = heat rigor; Ex = exhaustion.

Date, 1930.	Average weight of muscles, grammes.	Type of experi-ment.	Period of equilibration, hours.		Weight-ing factor.	Free water fraction.	Osmotic change, per cent. NaCl.	Lactic acid, per cent. of "free" water.
			Pre-liminary in R.	In 2R.				
24 Feb.	0·24	Ri	1	5½	1	0·82	—	—
24–25 Feb.	0·24	Ri	2½	14	2	0·83	0·37	—
26–27 ,,	0·27	Ex	0	19¼	4	0·79	—	—
27–28 ,,	0·26	Ex	0	17½	4	0·76	—	—
20–21 Mar.	—	Ri	2	17½	4	0·73	0·455	0·655
20–21 ,,	—	Ri	2	19	4	0·73	0·43	0·665
26–27 ,,	0·29	Ex	3½	15	5	0·79	0·44	—
27–28 ,,	0·22	Ex	1½	18	6	0·79	0·45	—
27–28 ,,	0·21	Ex	4	21	6	0·73	0·49	0·81
19–20 Mar.	—	Ri	4	(urea) 15	—	0·84	—	—
19–20 ,,	—	Ri	4	(urea) 16½	—	0·81	—	—

The mean value of the free water fraction, taking due account of the weighting factors, is 0·77; the mean value of the osmotic change is the equivalent of 0·45 p.c. NaCl.

allowed to diffuse out into the R-solution, the muscles would finally have been isotonic with 0·703 + 0·455 = 1·158 p.c. NaCl.

Two experiments were made with urea; they were good experiments, but the results are not included in the mean, for the reasons given above.

The experiments on fatigue were really experiments on rigor. Muscles severely fatigued will not long survive if deprived of oxygen; it was necessary, in order to make sure that equilibration was complete, to subject them to 15 hours or more of mixing; this means—since recovery would obviously have spoilt the experiment—a long period of oxygen want, during which they invariably passed into rigor. To ensure that the fatigued muscles should be alive at the end of it, equilibration would need to last not longer than about 4 hours. Only by using exceedingly small muscles, weighing, say, 50 mg. apiece (see fig. 2, p. 503), would it be possible so to quicken diffusion that

equilibration would be complete in that time. The difficulty of preparing a sufficient mass of such small muscles without injury was too great.

It is open, therefore, to any who will, to argue that the "free" water of *fatigued* muscles has not been measured at all. Strictly speaking this is so. The muscles could be properly described as fatigued during the earlier part of their equilibration, but during the latter part they were certainly in rigor. Since, however, the "free" water of muscles in rigor appears to be the same as that of muscles at rest, it is very unlikely that in fatigue, which in many respects may be regarded as an intermediate condition, the case is seriously different.

Fatigue was induced in two ways. In the first two experiments the intact legs were exhausted by induction shocks and then skinned; the muscles were dissected, placed immediately in the two tubes and weighed. In the last three experiments the muscles were dissected and soaked in Ringer's solution, blotted, placed in their tubes and weighed; they were then exhausted by induction shocks led through the contents of the two tubes in series. Finally in both cases the R and the 2R solutions were added. As before these contained NaCN, and the tubes were filled with nitrogen.

In three experiments the lactic acid production was measured. I am much obliged to Miss M. Kerly for making the determinations. The total lactic acid found in a tube is expressed in Table VII as a percentage of the initial "free" water of the muscle in the tube.

The mean value of the "free" water fraction for fatigued and rigor muscles, taking due account of the weighting factors, is 0·77. This is identical with the mean value for resting muscles. Again it may be slightly too low, owing to equilibration being not quite complete.

The osmotic change in fatigue and rigor has a mean value equivalent to 0·45 p.c. NaCl. The lactic acid actually produced, in the three experiments in which it was measured, averaged 0·71 p.c. calculated in the "free" water of the muscle. This is osmotically equivalent to 0·23 p.c. NaCl, which is 0·23 p.c. less than the mean osmotic change observed in the same three experiments. If we further suppose that all the phosphagen was broken down, liberating creatine equivalent to 60 mg. p.c. P, the extra osmotic effect would be equivalent to 0·07 p.c. NaCl. There is still an excess of osmotic pressure to account for, as found in the previous paper. This excess (equivalent to 0·16 p.c. NaCl) may seem rather small, when compared with that described there; actually its *absolute* value is almost exactly the same, but its *relative* size is diminished by the much greater amount of lactic

acid produced in the present experiments. We may recall also that in Table III, above, the vapour pressure depression caused by adding lactic acid to blood was shown to be appreciably less than that due to adding it to water; perhaps in muscle also the lactic acid is partly adsorbed by, or dissolved in, the solid constituents of the tissue. If so, the large amount of lactic acid produced in the rigor experiments would be osmotically equivalent in muscle to less than the amount of NaCl computed, and the unexplained excess of osmotic pressure still greater than calculated above.

The fundamental point brought out by the experiments of Tables VI and VII is that nearly the whole of the water of muscle is "free," in the sense that it can dissolve in a normal manner substances added to it. This is true, whether the muscles be at rest or in rigor. The mean value of the "free" water is 77 p.c. for both. The true value may be even slightly greater. It is important to define precisely what is meant by "free" water; the difference between the present results and those of others (*e.g.*, of Rubner) may be, in part at least, simply a matter of definition.

In dealing with the equilibria occurring in blood, or between blood and tissues, it is common to express concentrations in grammes (or mols) per litre, or per 1000 g. of tissue. It is clearly better, and likely to lead to much simpler relations, to express all concentrations (where physico-chemical equilibria are involved) in grammes (or mols) per 1000 g. of "free" water. If the "free" water be not accurately known, the total water is a close approximation, at least in muscles and blood.

V. The Swelling and Shrinking of Muscles in Hypo- and Hyper-Tonic Solutions.

The experiments of Overton (1902) have been referred to in the Introduction above. They have been repeated and extended as follows. The object was to measure the "osmotically active water fraction," *i.e.*, the weight of water per gramme of living muscle which may be regarded as surrounded by semipermeable membranes and liable therefore to osmotic increase or decrease in hypo- or hyper-tonic solutions.

It is necessary to consider for how long a time a muscle must be immersed in an aqueous solution of substances to which it is normally impermeable, in order to attain a sufficiently constant weight. The problem is one of diffusion, chiefly of water but partly also of salts, and is analogous to that discussed by Hill (1928, pp. 68–73, and fig. 5) in relation to the diffusion of oxygen into

a cylinder. The first stages of diffusion are rapid; the creep, however, to the final equilibrium is slow.

Fig. 1 illustrates the process graphically. Vertically is shown the change in weight of a cylinder composed of semi-permeable fibres, immersed in a hypo- or hypertonic solution, expressed as a fraction of the total change finally

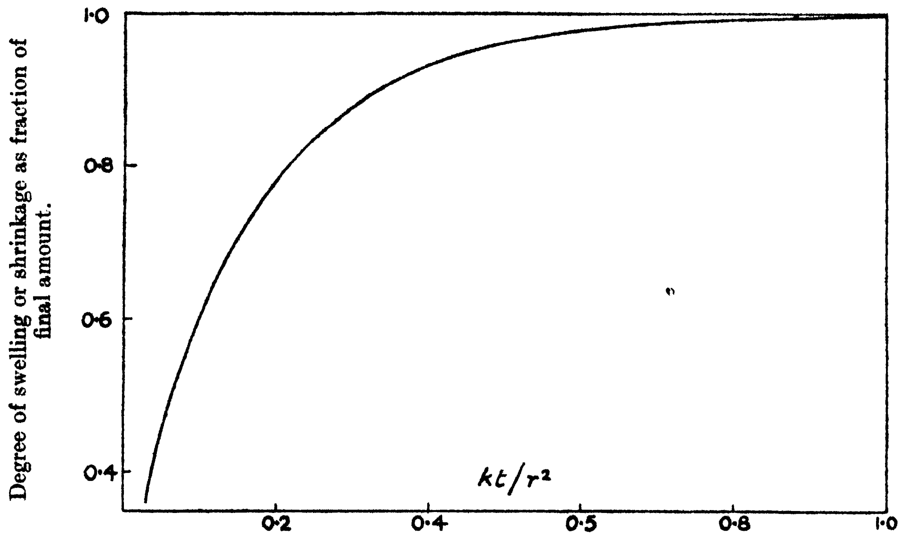

Fig. 1.—The swelling (or shrinkage) of a cylindrical tissue consisting of semi-permeable elements, when immersed in a hypotonic (or hypertonic) solution. Vertically, the degree of swelling (or shrinkage) as a fraction of that finally attained after long immersion; horizontally kt/r^2, where k is the diffusion constant of water in the tissue, t is time, and r is the radius of the cylinder. [Drawn from the data of Table IV, p. 71, of Hill (1928).]

attained after prolonged immersion. Horizontally is kt/r^2, where k is the diffusion constant of water into the tissue, t is the time, and r the radius of the cylinder. The curve was constructed from the data of Table IV in the paper just mentioned. The time needed for any given change is inversely proportional to the diffusion constant, and directly proportional to the square of the linear dimensions of the tissue; the factor kt/r^2 always occurs as a whole in dealing with a cylindrical tissue. Since the weight varies as the cube of the linear dimensions, for muscles of the same shape, we may conclude that the time required is proportional to the 2/3 power of the weight. As we shall see below, with muscles of the size used the time required to attain final equilibrium is so great that the values actually found are complicated by changes in the tissues. It is necessary to extrapolate from readings at an earlier stage.

It was thought desirable to avoid the use of solutions differing considerably in osmotic pressure from the muscles. The solutions employed were 0·8R and 1·2R, or 0·75R and 1·25R.* The muscles were always alive and normally excitable at the end of immersion. Since, however, progressive changes may occur in them, the simple procedure employed by Overton was not used, but a "differential" method was adopted. This automatically eliminates the effects of such unknown disturbances, each experiment being, so to speak, its own control.

The two gastrocnemii of a small frog were prepared and kept in oxygenated Ringer's solution (R) till required. Each was carefully blotted and weighed to the nearest 0·1 mg. on a Bunge air-damped balance. One was then hung in 0·8R solution, stirred by oxygen bubbling slowly through it, the other in 1·2R solution similarly stirred. After a sufficient period of immersion at room temperature the muscles were removed, blotted, and weighed to 0·1 mg. as before.

Consider a pair of similar muscles, *e.g.*, the two gastrocnemii of one frog. Let their initial weights be taken as unity, and assume that they have been brought, by soaking, into equilibrium with Ringer's solution R. Let (i) a be the solid plus "bound" water fraction; (ii) x the "osmotically active water fraction"; (iii) $(1 - a - x)$ the "osmotically inactive water fraction." Let us immerse one of the muscles in a hypertonic solution $(1 + r)$ R, and the other in a hypotonic solution $(1 - r)$ R, and leave them till a constant difference of weights sets in. Imagine that x changes in any way, owing to the processes of survival in salt solution, becoming $x(1 + \delta)$, where δ is positive or negative; causes of such change might be (1) a loss of semi-permeability in some of the membranes or fibres, in which case δ would be negative, or (2) a production of metabolites during survival, causing the tissue to swell, in which case δ would be positive, etc. Imagine further that $(1 - a - x)$ also changes as the result of immersion and survival, becoming $(1 - a - x)(1 + \delta)'$, where δ' is positive or negative; a cause of such change might be (1) an imbibition of water by the colloids of the tissue, or (2) a loss of semi-permeability in some of the fibres coming originally in the other fraction; in either case δ' would be positive. Let A be the final weight of the muscle in the hypotonic solution, B that of the muscle in the hypertonic solution. Since in the former the osmotic

* By a yR solution is meant, as before, an aqueous solution of the usual constituents of Ringer's fluid (except bicarbonate), in the usual relative proportions, but with $1/y$ of the usual amount of water.

pressure of the environment was changed from R to R $(1 - r)$, the "osmotically active water" must have increased in the ratio $1/(1 - r)$; hence

$$A = a + (1 - a - x)(1 + \delta') + x(1 + \delta)/(1 - r).$$

Similarly

$$B = a + (1 - a - x)(1 + \delta') + x(1 + \delta)/(1 + r).$$

We see at once from these formulæ the danger of making "single" uncontrolled experiments; δ' is unknown, it may be relatively large and positive, so that the apparent osmotic increase of volume in hypotonic solutions may be partly due to swelling in no wise caused by osmotic forces. That this danger is not imaginary is shown by the fact that in all the experiments performed on pairs of muscles, as described above, the increase of weight in the hypotonic solution was considerably greater than the decrease of weight in the hypertonic solution.

It is simple, however, to eliminate δ' completely by subtraction together with a; we find

$$A - B = x(1 + \delta)\, 2r/(1 - r^2)$$

so that the *final* "osmotically active water fraction" is given by

$$x(1 + \delta) = (1 - r^2)(A - B)/2r.$$

There is no direct way of finding the *initial* "osmotically active water fraction"; the best that can be done is to employ muscles so small that the time of equilibration is reduced as much as possible. For the two sets of observations to be reported first the gastrocnemii of the smallest frogs available were used. In the longer series of observations (by Kupalov) then described various muscles of widely different sizes were used. By extrapolation to "zero weight" the true value can be approximately estimated.

The following experiment is instructive. The 10 gastrocnemii of five frogs were dissected from 9.45 to 10.15 a.m., and left in oxygenated R-solution. From 11.15 to 11.42 a.m. they were blotted, weighed, and transferred, one set to oxygenated 0·8R-solution, the opposite set to oxygenated 1·2 R-solution. From 6.29 to 6.49 p.m. they were blotted and weighed again. They were soaked, therefore, in R-solution for 1½ hours, and in 0·8R and 1·2R for 7·17 hours.

Frog.	In 0·8R-solution.		In 1·2R-solution.		A–B calculated.
	Initial weight.	Final weight.	Initial weight.	Final weight.	
	mg.	mg.	mg.	mg.	
1	143·7	163·0	140·4	132·9	0·1876
2	246·4	268·0	253·0	239·2	0·1424
3	187·8	206·4	193·2	180·3	0·1659
4	213·5	229·8	202·3	188·5	0·1448
5	214·5	235·0	211·5	199·2	0·1539

It should be noted that A and B are fractions (final weight) ÷ (initial weight) in each case. The average value of (A – B) is 0·1589. The average initial weight of the muscles was 201 mg. Since in this case $r = 0{\cdot}2$, the "osmotically active water fraction" $(A - B)(1 - r^2)/2r$, is $2{\cdot}4\,(B - A) = 0{\cdot}382$.

In this experiment, however, there is evidence that a steady difference (A – B) had not been reached. On the same day a similar group of 10 gastrocnemii, soaked in oxygenated R-solution for 1·6 hours, was immersed, one set in 0·8R, the opposite set in 1·2R, for 6·16 hours. The average initial weight was 202 mg. and the average value of (A – B) was 0·1514. This is appreciably less (at 6·16 hours) than the value obtained for 7·17 hours. A third group of muscles, soaked in oxygenated R-solution for 1·7 hours, was immersed in 0·8R and 1·2R for 4·5 hours; the average initial weight was 194 mg. and the average value of (A – B), 0·1360. This is considerably less (at 4·5 hours) than the value at 7·17 hours. It is clear that a constant difference was not reached. We may, however, gain a rough idea of the true final value by extrapolation.

Our data are as follows :—

Time, hours	4·6*	6·16	7·17
A – B	0·1360	0·1514	0·1589

We have to choose three points on the curve of fig. 1 such that the abscissæ are in the ratio 4·6 : 6·16 : 7·17, and the ordinates in the ratio of the corresponding values of (A – B). If we take 4·6 hours as corresponding to $kt/r^2 = 0{\cdot}12$, then 6·16 and 7·17 hours correspond respectively to 0·161 and 0·187. The values of the percentage change read off from fig. 1 for these values of kt/r^2 are, 65·4, 72·6, and 76·5, from which the calculated final value of

* **4·6 hours is a corrected time, to allow for the fact that the muscles in this group weighed slightly less than in the others.**

(A — B) should be $0 \cdot 136/0 \cdot 654 = 0 \cdot 208$, $0 \cdot 1514/0 \cdot 726 = 0 \cdot 209$, and $1 \cdot 1589/0 \cdot 765 = 0 \cdot 208$, a satisfactory agreement. Thus the final value of (A — B) to which the muscles were tending is 0·208, which corresponds to an "osmotically active water fraction" of 0·50. To attain it, however, would have required about 24 hours, during which time the fraction in question would have diminished, owing to irreversible changes taking place in the muscles.

In another experiment 14 pairs of gastrocnemii, again averaging about 0·2 g. in weight, were dissected and left in oyxgenated R-solution all night at about 18° C. On the following day they were blotted, weighed and immersed in 7R/6 and 5R/6 solutions for 7¼ hours. Finally they were blotted and weighed again. The average value of (A — B) was 0·115, which corresponds to an "osmotically active water fraction" of only 0·336. The low value is doubtless due partly to too short a time of immersion in the experimental fluids, partly to a genuine decrease—caused by long survival—of the fraction of the muscle contained within functioning semi-permeable membranes.

A series of similar experiments was performed by my colleague Dr. P. Kupalov, on various muscles of the frog dissected on the same day. These were the gastrocnemius, the sartorius, the semi-membranosus, and several other muscles of the upper leg. The period of immersion was about 5½ hours, after a preliminary soaking of about 2 hours in Ringer's fluid; the solutions were either (*a*) 0·8R and 1·2R, or (*b*) 5R/6 and 7R/6. The muscles varied in weight from 30 mg. to 840 mg. Naturally with the smaller ones the experimental error was large, and moreover, in their dissection, there was risk of injury, which tended to increase the "scatter."

The results of these experiments are shown graphically in fig. 2, the value of the "osmotically active water function" being plotted against the initial weight of the muscle. The curve drawn through the points is a theoretical one, based on fig. 1, assuming the muscle to be cylindrical and r^2 (in kt/r^2) to be proportional to the 2/3 power of the weight. The extrapolated value for zero weight, viz., 0·47, is that to which, on the average, the muscles were tending at the time of observation (5½ hours). Apparently after 1 to 2 hours of preliminary soaking in Ringer's fluid and 5½ hours in the experimental solutions, the "osmotically active water fraction" is about 0·47; in other words, out of 0·77 g. of "free" water in 1 g. of muscle, 0·47 g. is confined within semi-permeable membranes and is subject to osmotic swelling or shrinkage in hypo- or hypertonic solutions, while 0·30 g. is unconfined.

Of the 0·77 g. of "free" water in 1 g. of muscle only a small part is present in the interspaces between the fibres; certainly far more than 0·47 g. is con-

tained within the fibres themselves. The reason why all the water is not "osmotically active" is not, as Overton supposed, that part of it is "bound";

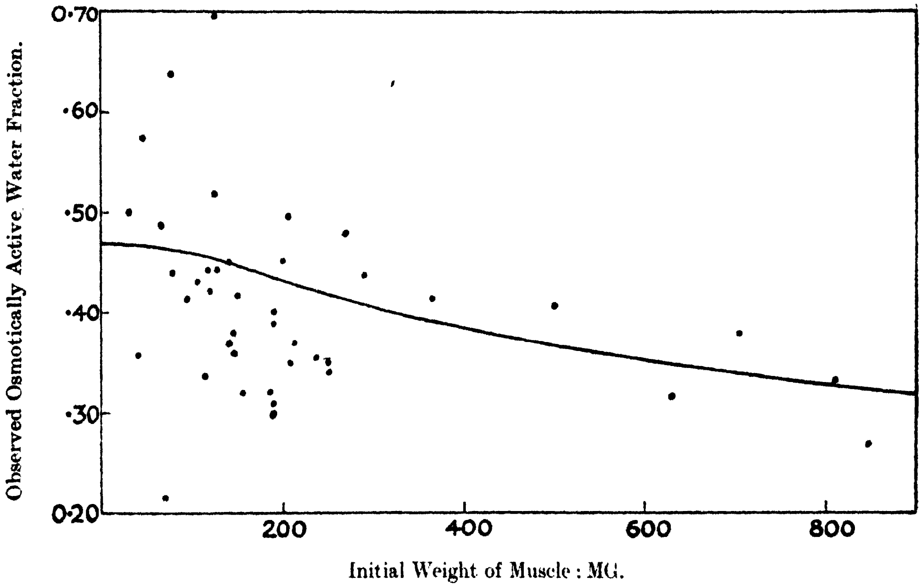

FIG. 2.—The "osmotically active water fraction" determined for a number of muscles of different sizes by the differential method described in the text, plotted against the weight. The curve drawn through the points was calculated from fig. 1, assuming (*a*) a constant time of immersion, (*b*) a constant coefficient k of diffusion, and (*c*) a cylindrical muscle, with r^2 varying as the 2/3 power of the weight. The true value, for the period of immersion considered, is that extrapolated to zero weight.

very little, if any, of it is "bound"; the simplest and most probable explanation is that, after prolonged survival without a normal blood supply, the membranes of some of the muscle cells have lost their semi-permeability. I am informed by Mr. H. V. Horton that a considerable fraction of its potassium may escape by diffusion from a muscle suspended in Ringer's solution, *e.g.*, 25 p.c. of it may be lost in 5 hours (see also Ernst and Scheffer (1928)). During life the membranes of a muscle are certainly impermeable to potassium. Apparently, however, the removal of their normal environment of blood plasma, or some other cause, somehow affects the membranes of a certain number of the fibres, rendering them permeable, allowing their K-ions to escape, and so making them presumably "osmotically inactive." The spontaneous onset of "reversible inexcitability" described by Dulière and Horton (1929) as occurring in an isolated surviving muscle is, so Mr. Horton informs me, almost

certainly caused by the escape of potassium from the inside of a certain number of its fibres; its reversal by washing is due simply to the removal of this potassium. If the original "osmotically active water fraction" were 0·64 (allowing 0·13 of the "free" water fraction 0·77 for water between and not inside the fibres), a loss of semi-permeability in 25 p.c. of the fibres after 7 hours survival would reduce the "osmotically active water fraction" to 0·48, which is about what we have found.

We may conclude, therefore, that, (*a*) progressive changes, owing to survival and immersion, (*b*) the slowness of diffusion, and (*c*) the loss of semi-permeability in a considerable fraction of the fibres, are the cause, and not any hypothetical "binding" of the water, of the relatively small osmotic effect of immersing muscles in hypo- or hypertonic salt solutions.

Summary.

1. Previous work on the state of water in biological fluids and tissues is discussed; it is pointed out that the various definitions of "free" and "bound" water do not necessarily coincide.

2. The "free" water fraction is defined as the weight of water in 1 g. of fluid or tissue which can dissolve substances added to it with a normal depression of vapour pressure: this is analogous to Gortner's definition, substituting "vapour pressure" for "freezing point."

3. To measure the "free" water of a fluid, a weighed quantity of some suitable substance is dissolved in a weighed quantity of the fluid, and the depression of vapour pressure measured, and compared with that caused by adding the same substance to an approximately isotonic salt solution.

4. To measure the "free" water of a portion of tissue, a weighed quantity of the latter is stirred for a sufficient time with a weighed amount of a hypertonic salt solution, and the change of vapour pressure measured.

5. The "free" water of blood, or of centrifuged corpuscles, is practically equal to the total water, being perhaps 2 p.c. less. In dealing with the equilibria occurring in blood (or muscle), concentrations should be expressed, not in grammes (or mols) *per litre*, but in grammes (or mols) *per* 1000 *g. of "free" water.* The osmotic pressure of blood is exactly accounted for by supposing all the known soluble constituents of blood to be freely dissolved in the "free" water.

6. The "free" water of casein solutions, or of concentrated egg white, is almost exactly equal to the total water.

7. The "free" water fraction of frog's muscle, whether resting or in rigor,

is about 0·77, or perhaps a little greater, the total water fraction being 0·80 or 0·81. Very little, if any, of the water of muscle is "bound."

8. The contrary conclusion, based upon Overton's experiments, is due to a variety of factors: (*a*) progressive changes caused by prolonged immersion in salt solutions; (*b*) the slowness of reaching diffusion equilibrium; and (*c*) the loss of semi-permeability in a considerable fraction of the fibres, as the result of removal from a normal environment.

9. The osmotic behaviour of muscles in hypo- or hypertonic salt solutions is considered.

I am indebted to Mr. P. Eggleton for advice and information, and to him and Mr. H. V. Horton for suggesting, making and allowing me to report the experiments with urea referred to in the text. I am indebted to Dr. P. Kupalov for making the experiments shown in fig. 2, and to Miss M. Kerly for the lactic acid measurements referred to in Table VII.

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The Use of n*th Order Equations to Describe the Action of Simple Hæmolysins.*

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Contents.

Introduction.

The purpose of this paper is to introduce a fundamental modification into the theory which deals with the kinetics of hæmolytic reactions. Hitherto it has been found sufficient to treat the reaction between the lysin and the cell component with which it reacts as one of the first order ; now, as a result of improved methods of investigation, it is necessary to treat it as a reaction of the nth order, *i.e.*, as one in which varying-sized groups of molecules may be imagined as reacting with one another. As a result of this modification, the equations hitherto used for describing the kinetics of simple hæmolysins become special cases of much more general expressions, which are not only able to describe hæmolytic phenomena over greater ranges with greater accuracy, but which are in all probability applicable to a much greater diversity of hæmolytic reactions.

I. *Methods.*

1. *Time-dilution Curves.*—In previous investigations it has been the practice to discontinue observations of the time required for complete lysis after from 45 to 60 minutes, the reason for this being two-fold: (*a*) that it is difficult to define end-points when the lytic process is slow, and (*b*) that very slight errors in the quantity of cells or of lysin in the system lead to great errors in the time for complete lysis, owing to the fact that the time-dilution curve, as it approaches its asymptote, runs nearly parallel to the time axis. The end-points over 60 minutes are accordingly apt to be unreliable and irregular, and in all previous researches we have ignored them by supposing that the asymptote of the curve may be placed only a little above the last observed point, *i.e.*, that the curve is rapidly approaching its asymptote after from 30 to 60 minutes. Recently, however, we have been able to make sufficiently consistent observations of the time for complete lysis up to times as long as 300 to 360 minutes, and the fallacious nature of the former treatment has become apparent; the time-dilution curves, in fact, can now be shown to rise above the lines which were formerly supposed to be asymptotes, while at the same time the simple equations of former papers have been found insufficient to describe the curves as now obtained.

Even if the desirability of continuing observations for more than 60 minutes is realised, it is by no means easy to define the end-points. It is, indeed, difficult even to describe the small differences in technique on which the success of the attempt depends, for these protracted observations can be made only with the most minute attention to detail and after much practice. In general, however, the technique employed is the same as previously described (1), but two small modifications are necessary.

(i) Since some of the hæmolytic systems require to be observed for periods of 5 or 6 hours, it is necessary to use a water-bath large enough to contain about 50 tubes at one time, for otherwise it is not practicable to plot a number of time-dilution curves in one working day; further, the temperature of the bath must be much more accurately controlled than hitherto. We use a Monel metal water-bath, 30 inches long, 11 inches deep, and 6 inches wide, stirred with jets of compressed air, and regulated to 0·02°; this bath is arranged as previously described, so that the tubes containing the hæmolytic systems are seen through glass walls against a ruled background lit by reflected light. The lower part of the bath contains a large copper spiral through which cold water can be circulated when the temperature of the bath is required to be less than

that of the room. As the loss of temperature which occurs when the warmed suspension is added to the warmed lysin is proportional to the difference in temperature between the room and the water-bath, the latter is kept in a room whose temperature can be adjusted between 20° and 30°; in some cases, when curves are being plotted at low temperatures, work is carried out in a refrigerator room which can be kept constant between 1° and 15°.

(ii) In observing the time required for complete lysis, at least two end-points may be selected; these differ by less than 1 per cent., but are nevertheless clearly distinguishable by eye. The first end-point is taken when the black lines of the background are clearly distinguishable through the tube containing the hæmolytic system; this corresponds to about 99 per cent. lysis. The second end-point is taken when the contents of the tube are perfectly clear and free from even slight cloudiness; this corresponds to complete lysis. As a rule it is immaterial which end-point is taken, so long as the observer is consistent; in plotting time-dilution curves up to 300 minutes, however, the second end-point must always be used if consistent values are to be obtained for the prolonged times. Neglect of this apparently trivial point results in serious under-estimation of the end-points for the higher dilutions of lysin and renders the subsequent calculations virtually valueless.

2. *Percentage Hæmolysis Curves.*—It is impossible to investigate the kinetics of hæmolytic systems fully unless the methods of measuring percentage hæmolysis are as satisfactory as the methods of obtaining time-dilution curves, since consideration of the latter curves alone may lead to quite misleading conclusions. Percentage hæmolysis curves of sufficient accuracy, however, are by no means easy to obtain. A number of methods, none wholly satisfactory, have been suggested, all utilising some method of measuring the intensity of light transmitted through the hæmolysing suspension. The radiometer method, proposed by one of us, requires a considerable quantity of cell suspension (20 c.c.), and is incapable of measuring rapid changes owing to the lag of the radiometer (2). The simple opacimeter method proposed by Jacobs, and also the ingenious method of the same investigator in which a glowing filament is matched against a background consisting of the hæmolysis suspension, both suffer from the disadvantage that they do not readily follow changes in the important range from 0 to 30 per cent. lysis. The method employing the selenium cell has this same disadvantage, and may possess an even more serious one in addition, for, as already stated, it is impossible to obtain consistent readings unless special precautions are employed to eliminate the effects of photoelectric fatigue (3). The extent to which this can be done

depends very much on the particular kind of selenium cell employed, and we have had such unsatisfactory experience with the various types of selenium cell on the market that we have abandoned the method for that described below, in which a potassium cell is used in place of selenium. In this connection may be mentioned the potassium cell method of Kesten and Zucker (4). This is a null method, and does not follow rapid changes satisfactorily; it is unsatisfactory in another respect also, for in our hands at least, it exhibits considerable instability, therein resembling most combinations of photoelectric cells with amplifying circuits.

The apparatus now employed is shown in fig. 1, and consists of a rectangular water-jacketed cell, of 3 c.c. capacity, interposed between a source of light and

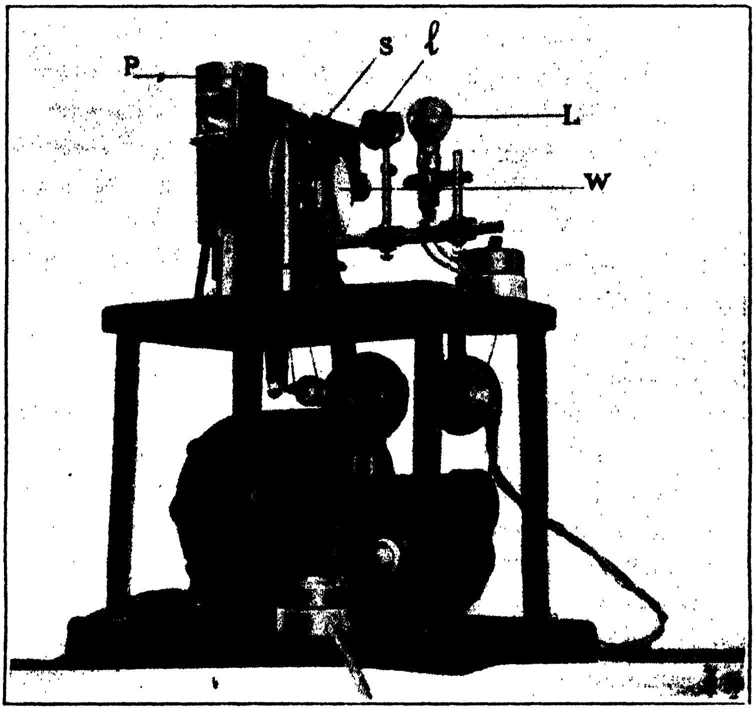

FIG. 1.—Apparatus for measuring percentage hæmolysis. L, light; *l*, lens; W, rotating wheel; S, slot, behind which is the water-jacketted cell containing the suspension: P, potassium cell in case.

a potassium photoelectric cell connected in series with a mirror galvanometer and a high-tension battery, the negative pole of which is earthed. The potassium cell used is one of the vacuum type, gas-filled cells being apparently much more unstable. It is surrounded by a light-proof shield containing a window,

1 cm. square, in line with the lower part of the glass cell and with the filament of the light. The light itself is a 6-volt projection lamp, lit from an accumulator. On no account must the source of light be operated on the ordinary laboratory electric supply, which is too inconstant for the purpose. A lens mounted rigidly on a small optical bench, which also supports the lamp, renders the light beam parallel and directs it through the cell suspension into the window of the photoelectric cell.

In order to obtain intermittent illumination of the photoelectric cell, which, like the selenium cell previously described, is best used in connection with a ballistic galvanometer, a rotating shutter is placed between the lens and the cell containing the hæmolytic system. This shutter, in the form of a wheel with an adjustable slot cut out near its periphery, can be made to rotate once every 15 seconds, 30 seconds, 60 seconds, or 300 seconds, and in this way the photoelectric cell is exposed to the light four times a minute, twice a minute, once a minute, or once every 5 minutes, for a period dependent on the length of the slot. Against the revolving shutter rest two light strips of copper connected to the terminals of the galvanometer ; these are adjusted in such a way that both touch the wheel, thus keeping the galvanometer short-circuited except during the time when the shutter allows the photoelectric cell to be illuminated ; when this illumination of the cell occurs, one strip fails to make contact with the wheel, and the galvanometer becomes undamped. The sequence of events accordingly is as follows :—The photoelectric cell is first unilluminated and the galvanometer is damped. The wheel rotates continuously. Just before the cell is to be illuminated the one copper strip fails to make contact ; the galvanometer becomes undamped, the photoelectric cell is immediately afterwards illuminated, and the extent of the swing of the galvanometer read. Just as the mirror swings back towards zero, however, the strip again makes contact with the wheel ; the galvanometer is at once damped, and almost immediately thereafter the shutter comes between the photoelectric cell and the light. The light is thus left on constantly and the photoelectric cell subjected to intermittent illuminations of equal duration and at equal intervals ; the fact that the exposures are made automatically is a great advantage, for, since a reading is taken at every rotation of the wheel, successive readings are 15, 30, 60, or 300 seconds apart, as the case may be.

The apparatus is used exactly as described in (3), the lysin and the components of the hæmolytic system (totally 1·6 c.c.) being placed in the glass cell and 0·4 c.c. of cell suspension, kept in a pipette in a thermos flask, being added at a suitable time. Readings of the galvanometer are made as they occur, and

are converted into values for percentage hæmolysis by use of a calibration curve. The hæmolytic system is kept stirred by a fine jet of compressed air; unless this is done, suspensions in the range of 0 to 20 per cent. hæmolysis may show variable light transmission. Kesten and Zucker, who mention this variable light transmission and regard it as constituting a serious difficulty, account for it on the grounds of crenation of the cells of the suspension; it is, however, entirely due to insufficient stirring, and can easily be eliminated.

In constructing the calibration curve, an important modification has been introduced. Previously we prepared the series of standards representing 0 per cent., 10 per cent., ..., 100 per cent. hæmolysis from the blood from which the standard cell suspension is prepared; we now prepare the same standards from the cell suspension itself. To prepare a 10 per cent. standard, for example, 0·1 c.c. of suspension is added to 4 c.c. of saline; the cells are then hæmolysed by freezing and thawing, and 0·9 c.c. of cell suspension added. The contents of the tube then exactly represented 10 per cent. hæmolysis in a hæmolytic system containing 0·4 c.c. of standard suspension in a total volume of 2 c.c. Other standards are prepared similarly, and the galvanometer reading corresponding to each obtained.

The last consideration is that of the precision which can be expected of the method in practice. Kesten and Zucker, for instance, regard their percentage hæmolysis readings, made by their potassium cell method, as correct to $\pm$ 0·5 per cent., but we think that such a precision, not only in their method, but in any method of measuring percentage hæmolysis, is quite unattainable. In the potassium cell method described above, for example, the precision is about the same as in the radiometer method or the selenium cell method, *i.e.*, $\pm$ 3 per cent. between 0 per cent. and 20 per cent., $\pm$ 2 per cent. between 20 per cent. and 60 per cent., and about $\pm$ 1 per cent. as complete lysis is approached. The errors are even greater when rapid hæmolysis is being investigated, for the exposure of the photoelectric cell is not instantaneous, and the degree of lysis thus increases even during the taking of each reading.

II. *Time-dilution and Percentage Hæmolysis Curves.*

In all recent work concerned with the fitting of formulæ to curves obtained for the action of simple hæmolysis it has been assumed that the "fundamental reaction" between the cells and the lysin is one in which the latter combines with some component of the former, thus forming a new compound, as a result of the formation of which the integrity of the cells is destroyed. Thus the quantity of cell component, S, destroyed is proportional to the quantity

of lysin, x, used up in the system, and the velocity of the reaction is given by

$$dx/dt = k(c - x) \tag{1}$$

whence

$$t = \frac{1}{k} \log \frac{c}{c - x}, \tag{2}$$

where c is the initial quantity of lysin, where t is the time required to produce lysis of an arbitrary number of red cells, and where S is large compared to c. Since it is assumed that the complete lysis of n cells corresponds to the utilisation of a constant quantity of lysin, we obtain, by putting x = const., and varying c in (2), a relation between the time for complete lysis of n cells and c the initial concentration of lysin; when plotted, this gives the time-dilution curve for the lysin. If we are concerned with the number of cells, N, hæmolysed from moment to moment by a particular concentration of lysin from the beginning of the reaction until its completion, we solve (2) simultaneously with an expression of the general type.

$$n = n_0 \int_0^x (1 + x/a_1)^{m_1} (1 - x/a_2)^{m_2} \, dx. \tag{3}$$

or with the integral of some other type of frequency distribution, and obtain the S-shaped percentage hæmolysis curves. (For all details of this earlier treatment, see (3) and (5).)

To show the way in which this treatment requires to be modified if protracted observations are made, we shall use a numerical example in which a time-dilution curve is obtained by the usual methods described above, the observations being extended to cover a period of 300 minutes.

(a) The following observations refer to a time-dilution curve for saponin acting on a standard cell suspension at 25° :—

Dilution, 1 in.	c, microgm.	t, experimental.	t, calculated.
10,000	200	0·5	0·76
20,000	100	1·3	1·76
30,000	66·6	3·1	3·09
40,000	50·0	5·6	5·4
50,000	40·0	12·5	13·0

The calculated results in the last column are obtained from expression (2) with $x = 39·0$, $k = 0·03125$, and agree quite well with the observed ones, although the excess of the calculated values over the observed values in the high concentrations is very apparent.

(*b*) By carrying out protracted observations as described above, the following values were obtained for the same lysin acting under the same conditions :—

Dilution, 1 in.	c, microgms.	t, experimental.	t', calculated.	t, calculated.
10,000	200	0·5	10·5	0·33
20,000	100	1·3	22·3	1·3
30,000	66·6	3·1	36·7	3·2
40,000	50·0	5·6	53·7	6·5
50,000	40·0	12·5	81·0	12·3
60,000	33·3	26·0	105·8	23·6
70,000	28·57	57·0	151·8	52·2
80,000	25·00	300·0	300·0	299·0

Hæmolysis accordingly occurs in dilutions of lysin in which, if the treatment in (*a*) is correct, it should never occur at all, and it is obvious that the asymptote at $x = 39 \cdot 0$ is not a true asymptote, and that the agreement of the calculated and the observed results in (*a*) is due to the fact that the latter are restricted. These results, indeed, cannot be described by a first order equation, as will be seen from the calculated values of t' shown in the fourth column of the table, which are derived from expression (2) with $x = 24 \cdot 4$ and $k = 0 \cdot 01244$.

In seeking a modification of expression (1) which will be sufficient to describe these experimental results without involving any great departure from the present theories, several possibilities present themselves.

(i) The velocity constant k might be itself a function of c, the concentration of lysin, instead of constant for all concentrations. This is not so unlikely as may appear at first sight, for k is a compound constant whose value is determined both by the "reactivity" and the quantity of the cell component which unites with the lysin, and it is quite possible that the mere placing of a cell in a certain concentration of lysin might alter its membrane in some manner dependent on the lysin concentration. If this were so, the hypothetical effect of various concentrations of lysin could be detected by immersing cells for a short time in the various concentrations, centrifuging, resuspending, and using the cells for the plotting of time-dilution curves which could be compared with curves obtained with cells not subjected to this preliminary immersion. Extensive experiments show that such preliminary immersion of cells in various concentrations of lysin produces no effects which support this hypothesis.

(ii) It is possible, as suggested in a previous paper, that the concentration of lysin which acts at the red cell surface is not proportional to the concentration c introduced into the system, *e.g.*, the lysin might be concentrated at the cell surfaces, and to a greater extent in some dilutions than in others. This

possibility must be discussed at some length, principally in relation to the views of Christophers (17), who, basing his conclusions on observations relating to the hæmolysis produced by various acids and bases in various concentrations, but extending them to the action of simple lysins in general, claims that a study of time-dilution curves, percentage ,hæmolysis curves, etc., do not elucidate the process of hæmolysis since the lysin is concentrated on, or "absorbed by" the cells of the suspension.

The facts upon which this view rests appear to be as follows: (*a*) if red cells are treated with comparatively small concentrations of acids, no lysis may result, but the acid may disappear almost completely from the supernatant fluid. As Christophers observes, it is taken up by the cells, in which it is presumably neutralised by intracellular buffers. (*b*) After some time (an hour or more) the cells so treated hæmolyse, although all the acid has been neutralised. (*c*) Even if the same cells are washed so as to remove acid from the supernatant fluid, hæmolysis occurs as if the washing had not taken place. (*d*) High concentrations of acid, on the other hand, produce rapid lysis before their neutralisation is complete or even extensive. These facts are quite well known, having been already described by Bodansky (18) and others. Christophers uses them, however, to support a view that hæmolysis cannot (even in the cases of lysins such as saponin and sodium taurocholate) be determined by the concentration of free lysin present in the system.

We may first inquire whether this conclusion is supported by the facts relating to lysis by acids and bases, and it is easy to see that these are susceptible of quite a different explanation. When the acid is present in high concentration, it acts, as Bodansky concludes, on the cell membrane: it probably behaves like a simple lysin, and the velocity of the action on the membrane is probably proportional to the concentration of free acid. The fact, however, that the acid can be neutralised by intracellular buffers makes its action unlike that of any simple lysin, for even when it is wholly neutralised the osmotic pressure within the cell is such that the cell volume increases (Hampson and Maizels (19)). The resultant stretching of an already weakened membrane thus produces a slow lysis, even although no free acid is present in the system. Only the action of unneutralised acid, accordingly, can be compared with the action of a simple lysin, the lysis which occurs after neutralisation is complete being due to quite a different process. Similar remarks apply to lysis by bases.

We may next ask whether there is any evidence of concentration of saponin, sodium taurocholate, sodium oleate, and similar lysins, on or within the red

cells. The answer may be supplied by three types of experiment :—(*a*) Cells are added to lysin in the usual way, and, after as long a time as can be allowed without hæmolysis commencing, are thrown down by a fast centrifuge. The concentration of saponin of lysin in the supernatant fluid is then determined. Experiments of this kind are very difficult, for the surface-active lysins are adsorbed on the glass of the tubes and of pipettes used for transference, but they indicate very clearly indeed that the lysin is not significantly concentrated on or within the cells. (*b*) The cells thrown down in the above experiments hæmolyse very slowly when the supernatant fluid has been removed, thus showing that the lysin in the supernatant fluid is involved in the lysis. (*c*) The sudden dilution of a hæmolytic system with saline, at any stage of the lytic process, delays hæmolysis, as it should do, by reducing the concentration of free lysin. All these experiments indicate that no measurable concentration of lysin occurs on or in the cells, and the results, together with the recognition of the fact that hæmolysis by acids and bases require a special treatment which allows for osmotic effects, justify us in setting aside Christophers' interpretations.

(iii) It is possible that the fundamental reaction, while being one in which the velocity is a function of the quantity of free lysin, is a reaction of more than the first order. This may occur in the following way. Suppose that the lysins concerned are not perfectly dispersed, but that they exist in the form of aggregates of molecules of varying sizes, as may be expected from their semi-colloidal nature. Then an aggregate of 1, 2, 3, ... molecules may react with each molecule of the cell component S, and if each such molecule of S requires the interaction of a number of lysins (say, 6, for example) it may obtain them by 6 additions of aggregates of 1, 3 additions of aggregates of 2, 2 additions of aggregates of 3, one addition of an aggregate of 6, or any one of a number of combinations of these possibilities. If, for example, the lysin existed in aggregates of 6 only, then the expression

$$dx/dt = k\,(c - x)$$

would describe the velocity of the reaction, as in (1), but if it existed in aggregates of 3 only, then

$$dx/dt = k\,(c - x)^2$$

would express the velocity. Thus, in general, if the lysin were to exist in aggregates of varying numbers, we should have the velocity given by

$$dx/dt = k\,(c - x)^n, \tag{4}$$

whence, if $1/p = n$,

$$kt = \frac{p}{p-1}\left\{c^{\frac{p-1}{p}} - (c - x)^{\frac{p-1}{p}}\right\}, \tag{5}$$

in which n or $1/p$ would be the value of the ratio (mean number of combining molecules)/(mean number of molecules per aggregate), and would have a meaning somewhat similar to that of the index n in Hill's equation for the dissociation of oxyhæmoglobin. The value of n, moreover, would in general be greater than unity, and would not, as a rule, be a simple integer.

Whether the manner of its derivation is correct or not, expression (5) has been found to describe closely the time-dilution curves of all the simple hæmolysis examined. In the case of the particular example given above, for instance, it provides the calculated time t shown in the last column, and these agree very closely with the experimental times over the entire range of the curve. The values assigned to the constants in this case were $x = 24 \cdot 4$ microgrammes, $n = 1 \cdot 8$ and $k = 0 \cdot 005952$. Other instances of the applicability of the expression will be given later on.

Considering next the percentage hæmolysis curves, it will be clear that these must be described by the simultaneous solution of (5) and (3), instead of (2) and another expression similar to (3), which may be called (3a), if the modification of theory is to hold. It would seem at first sight an easy matter to decide whether this is so or not. Provided, however, that we restrict ourselves to the investigation of the percentage hæmolysis curves for dilutions of lysins which complete lysins in 30 minutes or less, it is by no means easy (for technical reasons, among others) to detect the inadequacy of expressions (2) and (3a). This is best illustrated by giving a number of experimental and calculated

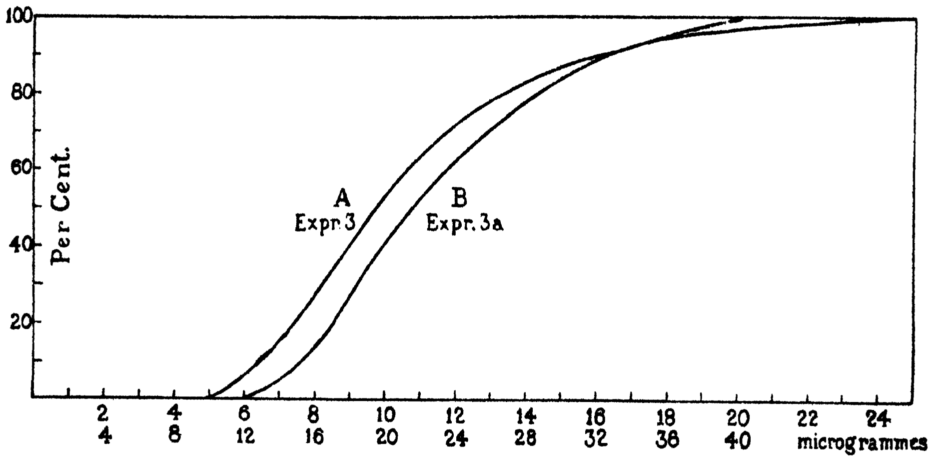

Fig. 2.—Integrals of frequency distributions, human red cells and saponin. For explanation see text. The upper scale on the abscissa applies to curve A, the lower scale to curve B.

percentage hæmolysis curves corresponding to the time-dilution curve used as an example above.

First we shall tabulate the data for the dilutions 1 in 30,000, 1 in 40,000, and 1 in 50,000. P is the percentage lysis observed at various times t. P (5) (3) indicates the percentage lysis calculated from expression (5) with $x = 24 \cdot 4$, $n = 1 \cdot 8$, and $k = 0 \cdot 005952$, together with a frequency integral described by expression (3), shown in fig. 2, A. P (2) (3a) is the percentage lysis calculated from expression (2) with $x = 39 \cdot 0$ and $k = 0 \cdot 03125$, together with a frequency integral shown in fig. 2, B, and described by an expression, denoted by (3a), of the same general type as (3).

1 in 30,000.

t	0·5	1·0	1·5	2·0	2·5	3·0
P	2	*55*	*80*	*91*	96	99
P (5) (3)	2	54	83	93	97	99
P (2) (3a)	0	25	64	85	94	99

1 in 40,000.

t	1·0	1·5	2·0	2·5	4·0	5·0
P	7	*37*	*65*	75	95	98
P (5) (3)	9	40	64	78	95	98
P (2) (3a)	6	25	55	72	93	98

1 in 50,000.

t	2·0	3·0	4·0	5·0	8·0	10·0
P	16	55	75	85	95	99
P (5) (3)	17	57	76	86	96	98
P (2) (3a)	17	57	76	86	96	98

The curious thing about these percentage hæmolysis curves is that, with the exception of the italicised observations, the values of P obtained could be used to support either the combination of (5) and (3) or of (2) and (3a), the sole difference being that the frequency curve used in conjunction with (5) would be more symmetrical than that used in conjunction with (2), as may be seen from fig. 2, in which the integrals of the two distributions are shown. Thus, to decide whether expressions (5) and (3) are preferable to expressions (2) and (3a) we would first have to decide that the frequency integral described by (3) is preferable to that described by (3a). This we cannot do on any *a priori* grounds.

Such comparatively restricted observations as these, accordingly, do not clearly indicate that the theory adopted in previous papers is inferior to the

present modification. Further, it is to be remembered that, in practice, even the italicised discrepancies might not arouse suspicion, for the curves rise very steeply, and quite a small error in timing either the commencement of the reaction or the moment of the reading would go far in the direction of abolishing them. In view of the fact that the galvanometer reading is not instantaneous, this is a very significant point.*

The percentage hæmolysis curves for the higher dilutions, however, show that expressions (2) and (3*a*) must be rejected in favour of (5) and (3).

1 in 60,000.

t	2·0	3·0	4·0	5·0	6·0	10·0
P	6	25	47	65	74	92
P (5) (3)	5	23	44	63	75	93
P (2) (3*a*)	6	32	50	67	75	91

1 in 70,000.

t	3·0	4·0	5·0	7·0	9·0	20·0
P	*7*	*23*	44	68	*80*	*95*
P (5) (3)	6	22	39	67	80	96
P (2) (3*a*)	15	36	49	66	—	—

1 in 80,000.

t	4·0	5·0	6·0	7·0	8·0	10·0
P	*5*	*17*	*32*	*40*	*51*	*64*
P (5) (3)	7	17	30	42	53	67
P (2) (3*a*)	15	40	—	—	—	—

None of the italicised observations could be accounted for by assuming a first-order reaction occurring in a heterogeneous population, identical to that postulated in the case of the higher concentrations, but all are consistent with the idea of the fundamental reaction being of the type expressed by (5) and the integral of the distribution of resistances being as shown in fig. 2, A.

* The discrepancies might not arouse suspicion for another, and perhaps more important reason, viz., that photometers embodying selenium or potassium cells are apt to be unreliable, as opposed to merely inaccurate in the sense that each reading has a considerable attached error. By this we mean that, although the apparatus may have, say, ± 3 per cent. error when working efficiently, this efficient working is dependent on so many factors, and may fall off so suddenly, that a very much larger error attaches to each reading if all possibilities of variation are to be allowed for. This unreliability naturally gives rise to a lack of confidence in the results, and one is always inclined to attribute unexpected observations to error, even if the discrepancies exceed the error which the method is known to possess when working efficiently.

Before leaving this example, it must be emphasised that the question of the most usual form of the frequency distribution of resistances is by no means disposed of. The correct distribution in the case of the above example presents a considerable negative skewness; in certain other cases, however, the distribution is much more symmetrical. Except for remarking that the form of the distribution is not only different in different animals, but also in members of the same species, little can be said until more extended observations are made, although the evidence at present points to an asymmetrical distribution with a negative skewness being the most common form. Some such cases are mentioned in (3). The distributions given by Kesten (6) and by Kesten and Zucker (4), if properly analysed, are negatively skew, although not nearly so skew as the former author thinks, nor as skew as the distribution whose integral is shown in fig. 2, A; most of the distributions given by Orahovats (7), on the other hand, are fairly symmetrical. Perhaps the most important point to be mentioned at present is that the use of a combination of two expressions such as (2) and (3*a*), in place of two such as (5) and (3), always results in the deduced distribution being more positively skew than the true distribution, especially if the value of n is high, *i.e.*, a negatively skew distribution appears more symmetrical, and a symmetrical distribution appears positively skew. The apparent symmetry of some of the curves reported in (3) may be due to this fact, and it is quite possible that a Pearsonian curve of type IV may always be necessary to describe the distribution of red cell resistances.

The above example illustrates excellently the reasons for considering the "fundamental reaction" to be a reaction of the nth order rather than one of the first order only, and it now remains, before passing to the consideration of the factors which bring about a variation in the value of n, to describe the simplest method whereby the value of the three constants in (5) can be found. The procedure is best illustrated by an example. Suppose, for instance, that we require to find the value of the constants for the following experimental time-dilution curve for saponin:—

δ.	t.	δ.	t.
10,000	0·75	60,000	14·3
20,000	1·0	70,000	47·0
30,000	2·1	80,000	60·0
40,000	3·8	90,000	180·0
50,000	9·7	100,000	600·0

The first step is to assign a value to x, the position of the asymptote. This is done by inspection, and in this case we can tentatively put $x = 19 \cdot 8$; this corresponds to a dilution of 1 in 101,000, and $\delta = 101{,}000$ is the highest ordinate of the curve. This ordinate is now regarded as 100 per cent. (much in the same way as we speak of the highest ordinate of an oxyhæmoglobin dissociation curve as 100 per cent.), and we proceed to find values of δ which correspond to 12·2 per cent., 24·4 per cent., 36·6 per cent., ... 97·6 per cent., as in the tables below.* The times corresponding to each such value of δ is now read off from the plotted time-dilution curve, and the results appear in the first three columns of the following table :—

Per cent. P.	Dilution.	t.	$\dfrac{t \text{ for } 85\cdot4 \text{ per cent.}}{t \text{ for P per cent.}}$
12·2	12,322	0·25	400·0
24·4	24,644	1·4	82·1
36·6	36,966	3·3	34·8
48·8	49,288	7·7	14·9
61·0	61,610	17·0	6·76
73·2	73,932	41·0	2·8
85·4	86,254	115	1·0
97·6	98,576	500	0·23
100·0	101,000	∞	—

The next step is to calculate the last column of the table, by dividing the time corresponding to 85·4 per cent. by the times for each of the other values of P. These values are then compared with the values in the horizontal columns of the following table, which show the values of the ratio (t for 85·4 per cent.)/(t for P per cent.) for the above value of P, and for all the values of n from 1·0 to 3·0.

* These particular values of P (12·2, 24·4, etc.) are used rather than values at more obvious intervals (*e.g.*, P = 10, 20, etc.) merely because we carried out our first complete calculations for the former.

P, per cent.

n	12·2	24·4	36·6	48·8	61·0	73·2	85·4	97·6
1·0	14·4	6·80	4·13	2·83	1·87	1·43	1·0	0·506
1·1	18·6	9·50	4·77	3·08	2·25	1·52	1·0	0·460
1·2	26·1	10·4	6·27	3·67	2·42	1·61	1·0	0·412
1·3	35·4	13·1	6·86	4·14	2·63	1·70	1·0	0·366
1·4	47·8	16·1	8·10	4·73	2·88	1·76	1·0	0·319
1·5	65·1	20·0	10·4	5·37	3·17	1·88	1·0	0·274
1·6	87·6	24·9	11·5	6·11	3·49	1·97	1·0	0·239
1·7	116	31·6	13·8	7·02	3·96	2·13	1·0	0·206
1·8	158	40·1	16·3	8·03	4·24	2·21	1·0	0·174
1·9	237	49·9	19·5	9·23	4·65	2·32	1·0	0·145
2·0	290	63·5	23·6	10·6	5·20	2·48	1·0	0·124
2·1	393	79·8	28·3	12·4	5·75	2·63	1·0	0·105
2·2	555	100	34·3	14·3	6·42	2·80	1·0	0·088
2·3	747	129	41·4	16·5	7·16	2·98	1·0	0·074
2·4	1064	162	50·0	20·0	7·94	3·17	1·0	0·061
2·5	1442	211	61·2	22·5	8·91	3·40	1·0	0·051
2·6	2004	267	74·5	26·1	10·0	3·64	1·0	0·043
2·7	2696	344	90·0	31·0	11·3	3·89	1·0	0·035
2·8	3863	499	111	36·7	12·7	4·17	1·0	0·029
2·9	5308	569	136	42·5	13·8	4·45	1·0	0·024
3·0	7929	793	168	50·4	15·9	4·78	1·0	0·019

The best agreement is seen to be in the column for $n = 2 \cdot 2$, which is accordingly selected as a sufficiently good value for this constant. We can now find how well the theoretical curve will fit the experimental values, by assuming that the point $\delta = 86{,}254$, $P = 85 \cdot 4$, $t = 115$ is correct, and then calculating the values of t from the theoretically correct values of the ratios shown in the column for $n = 2 \cdot 2$ in the table. This gives the following :—

P.	δ.	Ratio for $n = 2 \cdot 2$.	t, calculated.	t, experimental.
12·2	12,322	555	0·21	0·25
24·4	24,644	100	1·15	1·4
36·6	36,966	34·3	3·35	3·3
48·8	49,288	14·3	8·0	7·7
61·0	61,610	6·42	17·9	17·0
73·2	73,932	2·80	41·0	41·0
85·4	86,254	1·00	115·0	115·0
97·6	98,576	0·088	1307·0	500·0
100·0	101,000	—	∞	∞

With the exception of the value of the point for $\delta = 98{,}576$, situated at the extreme of the curve, the calculated and experimental values show a very close agreement, the greatest difference being only 0·9 minute. The discrepancy at the extreme end of the curve, however, may be disregarded in considering whether the fit is satisfactory or not, for the calculated value of this last point is extremely sensitive to changes in the value of x, while the experimental

value is apt to be unreliable; a discrepancy, accordingly, is to be expected, and can often be lessened by recalculation with a slight change in the value of x. The recalculation, in a case such as this, would not be undertaken without some special reason.

The last step in the fitting process is to find the value of k. This is done by inserting the values $t = 115$, $x = 19 \cdot 8$, $c = 23 \cdot 19$, and $p = 1/2 \cdot 2$ in (5) and solving for k. In this case the value found is $0 \cdot 0015$.

III. *Variations in n.*

The most important modification of the previous theory which has been made in this paper is the recognition that an expression of the nth order is more applicable to the "fundamental reaction" than is an expression of the first order, and it is therefore of particular interest to define the conditions under which changes in the constant n occur. Such conditions are doubtless very numerous, and their study as yet has been barely begun; four points, however, can be briefly mentioned: (*a*) the value of n obtained with different lysins; (*b*) the value obtained when different kinds of red cell are hæmolysed by the same lysin; (*c*) the effect of temperature; and (*d*) the effect of the addition of various substances to the hæmolytic system.

(*a*) Under comparable conditions there appears to be a value of n typical of the lysin employed. When a standard suspension of human cells is used, for example, the value of n, at 25°, usually lies between $2 \cdot 0$ and $2 \cdot 4$ for saponin, between $1 \cdot 5$ and $1 \cdot 8$ for sodium taurocholate, and between $1 \cdot 0$ and $1 \cdot 5$ for sodium oleate. The values vary somewhat with different specimens of the lysins, *e.g.*, we have in this laboratory two specimens of saponin, one of which gives a value of n of $1 \cdot 8$ with human blood, and the other of which gives a value of $2 \cdot 2$. In the case of the bacterial lysins, on the other hand, the value of n appears to be more nearly unity than in the case of most lysins examined.

(*b*) The results of a comparatively few observations indicate that even with the same lysin, *e.g.*, saponin, the value of n depends to some extent on the type of cell used in the hæmolytic system. In the case of saponin the variations seem to be small, at least where the observations are restricted to the cells of the ordinary laboratory animals. This follows at once from the fact that, although the asymptotes of the time-dilution curves vary considerably, the curves themselves do not cross, as they would necessarily do if n were to vary also (8). The cells of the camels, however, seem to constitute rather a special case, for Loo (9) finds that they are excellently fitted up to times as long as 70 minutes by expression (2); even if the use of (5) is preferable, n cannot be

greater than about 1·3, or it would be impossible to obtain such a good fit over so great a range. The saponin time-dilution curves for chicken blood, on the other hand, although fitted over a limited range by expression (2) (10), are best fitted by expression (5) with a value of n of about 2·0; the same remark applies to curves for reptilian çells, and so it appears that the fundamental reaction is quite generally of the nth order.

In the case of sodium taurocholate, however, n appears to vary considerably when the lysin acts on different cells, for the time-dilution curves for the cells of different animals cross very frequently, even though the position of the asymptotes varies less than in the case of saponin (8, 11).

(*c*) A full description of the way in which simple hæmolytic systems behave with temperature will be found elsewhere (12); in the meantime it is necessary to mention only the effect on the constant n. In systems containing saponin or sodium taurocholate, this constant significantly increases with temperature; in a system containing human red cells and the former lysin, for example, n = 1·3 at 5°, 1·4 at 10°, 2·4 at 25°, 2·6 at 30°, and about 3·0 at 35°. In the case of the latter lysin the increase with temperature is not so striking, but a change nevertheless occurs from about 1·1 at 5° to more than 2·0 at 35°. If the meaning given above to n is correct, this increase might result either from a diminution in the state of aggregation of the lysin or from a modification of the properties of the cell component which reacts with the lysin; further investigation, however, is required before any explanation of the result can be given.

(*d*) The effect of the addition of various accelerators and inhibitors on the value of the constant n is very variable indeed, and no general conclusion can be reached without extensive investigation. Some substances, such as glucose and other sugars, seems to have scarcely any effect at all, the value of n being the same, for example, in a cell-saponin-NaCl system as in one in which more than 50 per cent. of the NaCl is replaced by glucose. Other substances, such as certain electrolytes, have a considerable effect, of which the three time-dilution curves given below provide an illustration. The first is for a cell-saponin-NaCl system, the second for a cell-saponin-KCl system (in which the 0·8 c.c. of 0·85 per cent. NaCl of the first system is replaced by 0·8 of a KCl solution of the same tonicity), and the third for a cell-saponin-LiCl system.

P.	Cell-saponin-NaCl.		Cell-saponin-KCl.		Cell-saponin-LiCl.	
	t, observed.	t, calculated.	t, observed.	t, calculated.	t, observed.	t, calculated.
12·2	0·4	0·21	0·9	0·9	0·35	0·2
24·4	1·3	1·2	2·2	2·4	1·2	1·0
36·6	3·7	3·5	4·7	5·2	3·1	2·82
48·8	6·9	8·3	10	9·8	6·5	6·5
61·0	17	18·6	18·2	17·1	13·2	13·9
73·2	46	42·9	31·0	30·4	30	30·4
85·4	120	120	60	60	80	80
97·6	—	1454	240	251	—	761

For the first system, $x = 16 \cdot 7$, and $n = 2 \cdot 2$. For the second, in which KCl replaces part of the NaCl, $x = 13 \cdot 3$, and $n = 1 \cdot 6$. For the third, containing LiCl, $x = 11 \cdot 24$, and $n = 2 \cdot 1$.

Such variations, in the value of n where different electrolytes are used in the hæmolytic system are often met with, and are presumably the reason for the trouble which has been experienced hitherto in obtaining any general description of the effect of electrolytes in hæmolysis. The observation that n varies so considerably in such systems certainly provides an explanation of two facts : (*a*) that curves for systems containing different electrolytes frequently cross, and (*b*) that satisfactory values of R, the resistance constant, sometimes cannot be found for systems which differ much in electrolyte content.*

IV. *The R-notation.*

In previous papers (5, 15) a method was described whereby two hæmolytic systems, each containing the same lysin, can be compared with each other by the calculation of a resistance constant R. Where the fundamental reaction between cells and lysin is treated as one of the first order, this constant is derived in the following way. Suppose that one system contains saponin and red cells, and that the other contains saponin, red cells and an inhibitor such as sucrose. The time-dilution curve for the first system is

$$kt = \log \frac{c_1}{c_1 - x_1},$$

and that for the second is

$$kt = \log \frac{c_2}{c_2 - x_2}.$$

* Quite satisfactory R-values, however, may be obtained when two systems do not differ much in their electrolyte content (Kennedy, 14). This point is discussed in this paper.

The method is to compare concentrations which take equal times to produce lysis, and so we have

$$\log \frac{c_1}{c_1 - x_1} = \log \frac{c_2}{c_2 - x_2}.$$

Now suppose that the saponin-cell-sucrose system is R times as resistant as the saponin-cell system, *i.e.*, that R times as much lysin is needed to produce the same result. Then $x_1 = Rx_2$, and so

$$\log \frac{c_1}{c_1 - Rx_2} = \log \frac{c_2}{c_2 - x_2}, \tag{6}$$

from which it follows that

$$c_1 = Rc_2, \tag{7}$$

and that the result of plotting c_1, any concentration of lysin in the saponin-cell-sucrose system, against c_2, the concentration which produces lysis in the same time in the saponin-cell system, will be a straight line with a slope of R. This line, moreover, will terminate in a point where $c_1 = x_1$ and $c_2 = x_2$. The fact that this linear relation exists has been confirmed by a number of observers, and the above method of finding the resistance constant has been used not only in most of our own investigations, but also by Kennedy in his study of the effect of electrolytes (14), by Bodansky in his researches on the effect of pH (16), and by Loo (9) in his paper on the blood of the camels. It is accordingly very important to find the way in which (7) is modified when expression (5) is used to describe the time-dilution curves instead of expression (2), and when the position of the asymptotes is determined from observations lasting many hours instead of from observations lasting only 20 or 30 minutes.

Under these circumstances, the true asymptotes, which we shall call x_1' and x_2', will correspond to considerably higher lysin dilutions than do the "asymptotes" x_1 and x_2, which correspond very nearly to the dilutions of lysin which take about 30 minutes to complete hæmolysis. As before, $x_1' = Rx_2'$, but (6) now requires to be replaced by

$$(c_2 - x_2')^m - c_2^m = (c_1 - Rx_2')^m - c_1^m, \tag{8}$$

where $m = (p - 1)/p$.

When m is not an integer (*i.e.*, when n or p are not integers), an expression similar to (7) cannot be readily obtained from (8), but the general effect of the substitution of the reaction of the nth order for the reaction of the first order

can be seen from the expressions which correspond to (7) when $m = 1\cdot 0$ and $2\cdot 0$ respectively. In the first case $n = 2$ and

$$c_1 = \mathrm{R}c_2 \,.\, \frac{c_2 - x_2'}{c_1 - x_1'}. \tag{9}$$

In the second case $n = 3$, and

$$c_1 = \mathrm{R}c_2 \,.\, \frac{(2c_1 - x_1')\,(c_2 - x_2')^2}{(2c_2 - x_2')\,(c_1 - x_1')^2}. \tag{10}$$

When the reaction is of a higher order than the first, accordingly, the relation between c_1 and c_2 is represented, not by a straight line with a slope of R, but by a very flat curve in which c_1 is generally less than $\mathrm{R}c_2$; only at the point in which the curve terminates (*i.e.*, where $c_1 = x_1'$, and $c_2 = x_2'$) does this simple relation hold, for at all other points the relation between c_1 and c_2 is a function of the difference of these concentrations from the concentrations at the asymptotes of their respective curves, as well as of the order of the reaction, *e.g.*, of the ratio $(c_2' - x_2')/(c_1 - x_1')$, where $n = 2$.

The importance of these facts is (*a*) that all values of R hitherto obtained, since they have been calculated from observations restricted to about 30 minutes, require to be revised, but (*b*) that since the values c_1 and c_2 obtained when $t = 30$ minutes (about) bear an approximately constant relation to the values which would be obtained when $t = 300$ minutes the values of R hitherto obtained probably deviate from the true values in an approximately constant manner. The reason for the existence of this approximately constant relation is not difficult to appreciate if an actual example is considered. Consider two time-dilution curves described by (5), in one of which a cell-saponin system $x = 20$ microgrammes, and in the other of which a cell-saponin-aspartic acid system $x = 10$ microgrammes. In each, $n = 2$ and $k = 0\cdot 01$. The following table shows t_1 and t_2 the times for complete lysis in the two systems respectively :

δ.	t_1.	t_2.	δ.	t_1.	t_2.
10,000	0·6	0·2	80,000	160	27
20,000	2·5	1·0	90,000	400	38
30,000	6·5	2·0	100,000	—	50
40,000	13·0	5·0	120,000	—	91
50,000	25·0	8·0	140,000	—	163
60,000	45·0	13·0	160,000	—	320

By plotting the curves and reading off values of c_1 and c_2 corresponding to equal values of t, we obtain the following table :—

t.	c_1.	c_2.	c_1/c_2.
0·6	200·0	125·0	1·60
2·5	100·0	62·5	1·60
6·5	66·6	43·5	1·53
13	50·0	33·3	1·50
25	40·0	26·0	1·53
45	33·3	20·8	1·60
160	25·0	14·3	1·74
320	22·7	12·5	1·82
∞	20·0	10·0	2·00

These values of c_1 and c_2 are plotted against each other in fig. 3, A, and can be seen to lie on a curve so flat as to be almost a straight line. This curve

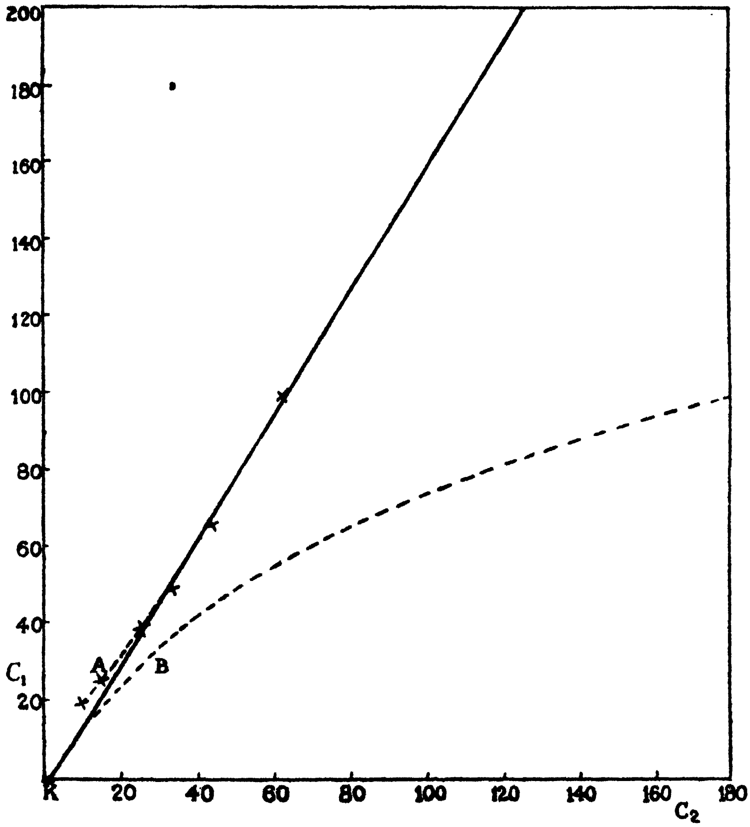

FIG. 3.—For explanation see text.

terminates where $c_1 = x_1' = 20$, and $c_2 = x_2' = 10$, *i.e.*, the value of R in (9) is $x_1'/x_2' = 2 \cdot 0$, and calculation will show that c_1 is always related to c_2 in the manner described by (9). If observations are restricted to the first 20–40

minutes, on the other hand, and if (7) is used on the supposition that $n = 1$, the points will be supposed to lie on a straight line whose slope is approximately 1·6, and this value will be assigned to R in (7), whereas the true value, obtained from the true asymptotes x_1' and x_2', is 2·0. Provided the observations are so restricted, however, expression (7), with a value of R of 1·6 will describe the results excellently, except that account will require to be taken of the intercept on the c_2 axis, expression (7) being replaced by $c_1 = \mathrm{R}\,(c_2 - k)$, which, to avoid confusion, we shall now write

$$c_1 = \mathrm{C}\,(c_2 - k), \tag{11}$$

and the value 0·002 being assigned to k. The straight line drawn through the points derived from this limited range of observations, however, does not pass through the point where $c_1 = 20$ and $c_2 = 10$, but terminates in a point corresponding to the two supposedly asymptotic concentrations, *i.e.*, two concentrations a little less than those which produce lysis in a time of from 20 to 40 minutes. In this case the terminal point would be where $c_1 = 30$, $c_2 = 20$, from which $c = c_1/c_2 = 1 \cdot 5$, approximately the same value as for the slope of the line.

This example will serve to illustrate three general conclusions which can be drawn from it and similar cases :—

(i) If observations are restricted as they have been in all previous investigations, and if the time-dilution curves are treated as those resulting from a first order reaction, instead of from a reaction of a higher order, the value of R (or of C) obtained when the two systems are compared by plotting c_1 against c_2 is always less than the true value. It is impossible, however, to say how much less the resistence will appear, for the straight line drawn through the experimental points really passes through points which fall on a curve ; its slope, accordingly, has very little real meaning, for if it passes very closely through the points in one part of the experimental range, it must pass less closely through points in other parts, its exact position, in fact, being rather arbitrarily susceptible of adjustment. The values of R obtained in previous investigations are thus certainly too small, but the general validity of arguments based on the values nevertheless remains much the same, for a detailed investigation would probably show that C, the slope of the line drawn through the values of c_1 and c_2 for the limited range, is quite a simple function of R, the relative resistance of the system as judged by the ratio of the true asymptotes of their time-dilution curves. Comparison of expressions (7) and (9), indeed, suggest that, when $n = 2$, R is approximately proportional to C^2.

(ii) The fact that the plotting of c_1 against c_2 results in a flat curve where the fundamental reaction is of a higher order than the first accounts for the appearance of the small intercept k referred to in expression (11). This constant has always been treated as an empirical constant, and an attempt has been already made to show that it might arise from the relation between c_1 and c_2 not being truly linear (5); the above consideration, however, makes the lack of linearity quite clear, and shows that the constant k arises as a result of using a straight line to approximate to the curve.

(iii) It has always been difficult to explain the fact that, even when great quantities of an inhibitor or an accelerator are used in a hæmolytic system, the value of R found, by treating the lytic reaction as one of the first order and using expression (7) or (11), is never greater than about 3·0 or less than about 0·3, *i.e.*, added substances appear unable to vary the resistance more than tenfold, whereas a much greater variation might reasonably be expected (13, 15). An extreme case arises when glucose is used as an inhibitor, for, if a system containing 2·0 c.c. of 0·85 NaCl has a resistance of unity, a system containing 1·959 c.c. of isotonic glucose and only 0·041 c.c. of 0·85 NaCl has an R value of 3·0; a system containing no electrolyte at all, on the other hand, has probably an infinite resistance, and one would expect the removal of 98 per cent. of the electrolyte to result in the resistance being increased more than three times. It has been shown above, however, that the values of R obtained in these earlier investigations are too low; the effect of added substances must, therefore, be greater than has been supposed, and it is probable that, when the investigations are repeated by improved methods, the hitherto inexplicable failure of most substances to alter the resistance more than slightly will be shown to be due to the use of a faulty method of analysing the results.

Finally, there remains to be considered a case which has been already mentioned, in which the addition of an accelerator or an inhibitor to a hæmolytic system alters both the resistance of the system (as judged by the position of the asymptote) and the order of the reaction, *i.e.*, the value of n. Under these circumstances, unless the change in the value of n is quite small, the linear, or almost linear, relation between c_1 and c_2 disappears altogether, and c_1 is related to c_2 by a curve. Such a curve is shown in fig. 3, B, the data being derived from the experiment, quoted earlier in this paper, in which NaCl is replaced in a hæmolytic system by KCl. The effect of the replacement is to decrease the resistance of the system from unity to 0·83, and at same time to change the value of n from 2·2 to 1·6; as a result, the relation between c_1 and c_2 is curvilinear, and such that it would be impossible to draw any straight

line which would pass even approximately through the points. This at once indicates that the value of n has changed; indeed, if a straight line can be drawn through the points with reasonable accuracy, and a reasonably constant value of R obtained therefrom, one may be certain that no great variation in n has occurred.*

As may be judged from the frequency with which satisfactory R-values are obtained when accelerators and inhibitors are added to hæmolytic systems, extensive changes in the order of the reaction are not very common. The addition of electrolytes may bring about such changes, as has already been mentioned, but perhaps the best example of the failure to obtain R-values as a result of a change having occurred in the value of n is the case in which an attempt is made to compare hæmolytic systems at different temperatures. In such cases the relation between c_1 and c_2 is usually curvilinear, and no value can be assigned to the resistance constant except by expressing the value of x_1/x_2 obtained from the position of the asymptotes.

V. *Summary.*

When the kinetics of simple hæmolytic systems containing saponin, sodium taurocholate, or other simple lysins, together with washed mammalian red cells, are investigated by improved methods which are described in this paper, the results are found to be best described by an expression of the nth order instead of by one of the first order ("monomolecular"), taking place in a population of cells whose resistances are distributed according to one or another type of frequency curve. The first order reaction which has hitherto been postulated describes the result quite well over the limited range which has been investigated hitherto, but is based on assumptions which are seen to be wrong when the experimental range is sufficiently extended.

The value of n determining the order of the reaction which is now supposed to be proceeding varies between about 1·0 and about 3·0, the most usual values, at ordinary temperatures, being between 1·5 and 2·0. The value of n varies, however, with the nature of the lysin, with the temperature, and probably with the type of cell, and is also affected by the addition to the hæmolytic system of some accelerators and inhibitors. It is tentatively suggested that the value of this constant is determined by the lysin existing in aggregates of varying size, and by the lysin reacting with some cell component, the molecules of which present varying affinities for the lysin molecules;

* If the change in n is very great, the relation may appear linear once more, but the slope and position of the line are such as to show immediately what has occurred.

even if this suggestion proves to be incorrect, however, the equation developed from it are useful in that they provide a very complete description of the phenomena.

The type of frequency curve which describes the distribution of red cell resistance to the lysin is probably always negatively skew, the degree of skewness, however, varying considerably. In previous papers the distribution has been supposed, in many cases at least, to be of a symmetrical type, but it is probable that this symmetry is spurious, and the result of treating the reaction between cells and lysin monomolecular, when it is really of a higher order.

While the treatment of the reaction between cells and lysin as one of a higher order than the first constitutes quite a fundamental change in the theory of the kinetics of simple hæmolysins, it is shown that the conclusions of most previous investigations require no essential modification. On the other hand, quite a number of observations, inexplicable on the previously adopted theory, become readily explicable on the newer hypothesis.

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The Effect of Variations in the Cell Content of Hæmolytic Systems.

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Several years ago, in order to explain the fact that the quantity of lysin apparently used up in producing complete lysis does not increase as much as might be expected when the number of red cells in the system is increased, I introduced the idea of a localised action of the lysin at the red cell surfaces, only those molecules of lysin near the cell surface, and contained within a hypothetical "zone of action" surrounding the cell, being supposed to take part in the hæmolytic process (1). A later investigation (2) brought out the interesting fact that the volume of the zone of action is approximately proportional to the resistance of the particular type of cell studied, *e.g.*, the zone surrounding each cell in a system containing saponin and sheep cells is about five times as large as in a system containing the same lysin and rabbit cells, the resistance of which is only about one-fifth of that of the cells of the sheep. The methods used to test the validity of the hypothesis were, however, much less satisfactory than those at present available. I feel, accordingly, that the effect of varying the cell content of hæmolytic systems should be investigated afresh particularly for two reasons: (*a*) that hæmolytic reactions are now believed to be of an order higher than the first, whereas my earlier analysis treated them as reactions of the first order, and (*b*) that my earlier experiments covered a smaller range of cell concentrations than is desirable.

I. *Theoretical Considerations.*

Let us suppose that a hæmolytic system of volume V contains n cells, and that another n cells are added to it, so that the total cell content is $2n$. As has been shown in (1), each one of the n cells originally contained in the system may be supposed to possess its own "zone of action" of volume v'; the chance of an added cell falling within one of these zones is accordingly nv'/V. That portion of the added number n which so enters the zones established round the previously contained n cells will form an "increment," in the sense that they will cause a delay in the complete lysis of the cells within whose zones they

fall, but that portion of the added number which falls outside the zones of action will not be perceived as an increment; in so far as the first number is concerned, they might not have been added at all, since they occupy regions of the hæmolytic system which contain lysin molecules which do not react with the cells first considered.

Suppose that we define the resistance of the system containing n cells in the usual way, *i.e.*, as the quantity of lysin x_1, or $c_{1\infty}$ transformed when lysis is complete. Let us also denote the resistance of the second system, containing $2n$ cells, by x_2 or $c_{2\infty}$. Then, since the amount of lysin transformed is proportional to the number of cells, we can write the resistance of the second system relative to that of the first as

$$\mathrm{R} = \frac{c_2}{c_1} = \frac{n+i}{n}, \tag{1}$$

where i denotes the "increment." If all the lysin in the system were involved at one time, i would be equal to n; when zones of action of individual volume v' exist, however,

$$i = \frac{n \,.\, nv'}{\mathrm{V}}. \tag{2}$$

These two expressions define, at least approximately, the relation between the values of c_∞ and of n in the various systems, for if we have suspensions of cell content n, $2n$, $4n$, etc., the corresponding values of c_∞ will be

$$\left.\begin{array}{l} c_0\,(1 + nv'/\mathrm{V}), \\ c_0\,(1 + nv'/\mathrm{V})\,(1 + 2nv'/\mathrm{V}), \\ c_0\,(1 + nv'/\mathrm{V})\,(1 + 2nv'/\mathrm{V})\,(1 + 4nv'/\mathrm{V}) \end{array}\right\}, \tag{3}$$

etc., where c_0 is the value of c_∞, obtained by extrapolation, where $n = 0$. If we neglect terms of the second order, this result may be written in the form

$$c_\infty = c_0\,(1 + k\,(2n - 1)), \tag{3A}$$

where k is a constant. The value of c_∞ is thus very nearly linear with the value of n over the upper part of the experimental range, as assumed in (1). By the use of expressions (1) and (2) the value of v', the volume of the individual zone of action, can easily be obtained from the values of c_∞ for two values of n, the second of which is twice the first.

Further, by means of these relations we are able to compute, from the time-dilution curve of a system containing any specified number of cells, the entire time-dilution curve of a system containing any other number of cells, but

otherwise similar. As has already been shown, both time-dilution curves can be described by the expression

$$kt = \frac{p}{p-1}\left\{c^{\frac{p-1}{p}} - (c-x)^{\frac{p-1}{p}}\right\}, \tag{4}$$

and their form is accordingly completely defined if the value of three constants, k, p and x, are known. If we call the constants relating to the time-dilution curve for the system with the smallest number of cells k_1, p_1, and x_1, and those relating to the system with the larger number of cells k_2, p_2, and x_2, then the following relations between the constants must follow from the above considerations :—

$$\left.\begin{aligned} x_2 &= x_1 + \psi x_1 \\ p_2 &= p_1 \\ k_2 &= k_1 + \psi k_1 \end{aligned}\right\}, \tag{5}$$

where ψ is a constant. The value of all three constants can be evaluated from the time-dilution curves by the method described in the preceding paper, and, by comparing the constants for two time-dilution curves containing different numbers of cells, it is easy to test the hypothesis under consideration.

II. *Methods.*

The values of c_∞ for suspensions of varying cell content, from which the successive values of R are to be calculated, may be obtained by plotting time-dilution curves in the usual way, but extending the observations up to 300 minutes or more. This procedure, however, is very laborious, and may conveniently be replaced by the following :—A series of saponin dilutions in 0·85 per cent. NaCl are prepared in the usual way. Of each dilution 0·8 c.c. is placed in a tube, 0·8 c.c. of 0·85 per cent. NaCl is then added, and the mixture is shaken. To the mixture is added 0·4 c.c. of the cell suspension whose resistance is being investigated. The hæmolytic system is then kept for from 7 to 9 hours at constant temperature, with vigorous shaking every 15 minutes. At the end of this time the highest dilution of lysin which has produced complete lysis is noted, and the smallest concentration of lysin (in microgrammes) capable of producing complete lysis in 7 to 9 hours is accordingly found. Such a time is sufficiently long to permit us to treat this concentration as c_∞, the concentration of lysin corresponding to the asymptote of the time-dilution curve. A similar series of lysin dilutions are put up for each concentration of cell suspension used, and thus a series of values of c_∞, corresponding to suspensions containing different number of cells, are obtained.

For the sake of convenience, the number of cells in these suspensions should be multiples of the number contained in a "standard" suspension (the thrice washed cells of 1 c.c. of blood suspended in 20 c.c. of 0·85 per cent. NaCl), and this number should be accurately known. In the most complete form of experiment, suspensions 8 ×, 4 ×, 2 ×, 1 ×, 0·5 ×, 0·25 ×, 0·125 ×, and 0·0625 × the standard suspension, are used. Suspensions stronger than eight times the standard cannot be used, for the difficulty in reading the end-point corresponding to complete lysis becomes very great indeed. An even greater difficulty appears when the suspensions are dilute (*e.g.*, 0·25 × to 0·0625 × standard) for if the number of cells is less than about a quarter of those contained in the standard suspension (*i.e.*, if there are less than about $2 \cdot 5 \times 10^7$ cells in the hæmolytic system of 2·0 c.c. volume) it is impossible to define the end-points accurately with the unaided eye, and it is necessary to have recourse to an apparatus which enables even exceedingly slight opacity in a hæmolytic system to be detected.

The instrument, which I call a "high-dilution apparatus," consists of two horizontal microscopes, 20 cm. long, mounted facing one another on an optical bench. Each can be moved by a rack and pinion, and each has a 25-mm. objective and a 9 × eyepiece. Between the two objectives is a series of 5 glass chambers, each 4 cm. long, 1·2 wide, and 1·2 cm. deep; any one of these chambers can be moved by turning a milled head, so that it lies between the microscope objectives and in their optical axis. About 7 cm. from the eye-piece of one microscope is a frame which holds a piece of white matt paper; behind this is a light. (See fig. 1.)

The apparatus is used in the following way. One of the five chambers is filled with saline, and the objective of the observing microscope is brought to within a few millimetres of its face. The other microscope is then moved until a sharp image of the surface of the matt screen is seen through the observing microscope. The image of the screen, under these circumstances, is first formed in the fluid in the chamber, and about 22 mm. from the face nearest the observing objective; it will be plain that if the fluid in the chamber contains even a slight opacity, this image will not be clearly seen. When the glass chamber is occupied by a hæmolytic system, accordingly, the appearance of the clear image of the screen in the observing microscope may be used as a criterion of complete hæmolysis. The five chambers enable several hæmolytic systems to be examined in succession, or standards, representing 99 per cent. or complete lysis, to be compared with the hæmolytic systems. By means this apparatus it is quite easy to detect the difference between 98 per cent.

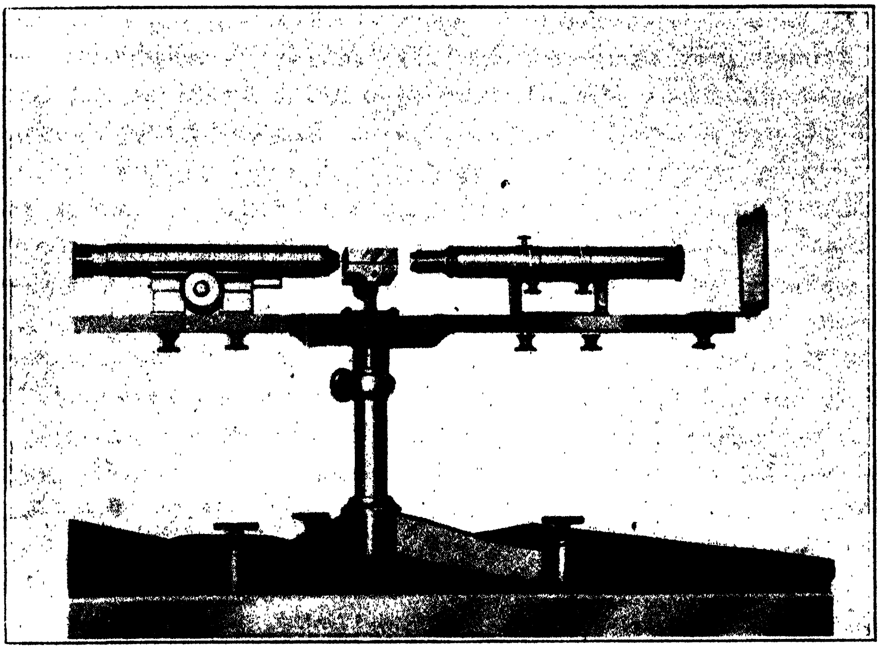

Fig. 1.—High-dilution apparatus. For description see text.

and complete lysis of a suspension one-sixteenth the strength of the standard.

In carrying out these experiments with suspensions of varying concentrations, care must be taken that the series of dilutions of lysin progresses by suitably small steps; a good rule is that the quantity of lysin in each tube shall be about 20 per cent. less than that in the one immediately preceding it. As a rough interpolation between two tubes is always possible, the value of c_∞ can thus be found with a maximum error of less than $\pm$ 10 per cent., *i.e.*, the value of $c_{1\infty}/c_{2\infty}$, or R, can be found with a maximum error of the same magnitude. Under the conditions of the experiments, such an accuracy is as high as can be expected.

III. *Results.*

These will be considered under three heads:—(*a*) the verification of the predicted relation between c_∞ and n; (*b*) the evaluation of the radius of the zones of action for various types of cell when acted on by various hæmolysins; and (*c*) the verification of the relations between time-dilution curves contained in expression (5).

(*a*) *The Relation between c_∞ and n.*—In the following table are shown the

values of c_∞ obtained for hæmolytic systems containing saponin and various numbers of (i) rabbit cells, and (ii) sheep cells, these two types of erythrocyte being selected because of their greatly differing resistance. The value of n gives the number of cells in the 2·0 c.c. volume of the hæmolytic system; c_∞ is expressed in microgrammes, and the values found may be compared with the values calculated, the latter being obtained in the following way. Each observed value of c_∞ is divided by the value before it (*i.e.*, for half the number of cells), and in this way a value of R and thence one of (R — 1) is found. By division of the latter by the number of cells in the more dilute suspension, we obtain v'/V, which should have a constant value; in practice, however, it never has, so we use its mean value to calculate the predicted values of c_∞.

Suspension in terms of standard.	$n \times 10^{-8}$.	Rabbit.		$n \times 10^{-8}$.	Sheep.	
		c_∞ observed.	c_∞ calculated.		c_∞ observed.	c_∞ calculated.
0·0625	0·075	4·03	4·035	0·108	26	25·82
0·125	0·15	4·10	4·105	0·217	28	27·52
0·25	0·3	4·25	4·248	0·435	31	31·15
0·5	0·6	4·55	4·544	0·87	38	39·37
1·0	1·2	5·2	5·177	1·74	60	60·15
2·0	2·4	6·74	6·620	3·48	120	123·7
4·0	4·8	10·5	10·31	6·96	260	—

In the case of the rabbit cells, the value of c_0 is 4·00 microgrammes (this being obtained by graphical extrapolation to where $n = 0$; see fig. 2), and the mean value of v' is 4646 μ^3; in the case of sheep cells, $c_0 = 25$ microgrammes and the mean value of v' is 12,120 μ^3. The results are plotted in fig. 2, and it will be observed that the agreement between the calculated and the observed values of c is very good indeed. The agreement may, I think, be fairly taken as evidence that the underlying theory corresponds, formally at least, with the realities of the situation.

Two points require comment:—(i) If the number of cells in the hæmolytic system is very small indeed, the cells with their surrounding zones may be so far separated that, even if the number is doubled, the chance of any of the added cells entering previously existing zones is so small as to be negligible. To obtain an idea of the number of cells in such a system, we can put $n \, . \, nv'/V = 1$ in expression (2), and solve for n by inserting known values of v' and V; *e.g.*, for rabbit cells a representative value of v'/V is $2 \cdot 5 \times 10^{-9}$, and so n

works out at about 2×10^4 cells. If the number of cells is reduced below this figure, the value of R will not change at all, for all the zones of action will be

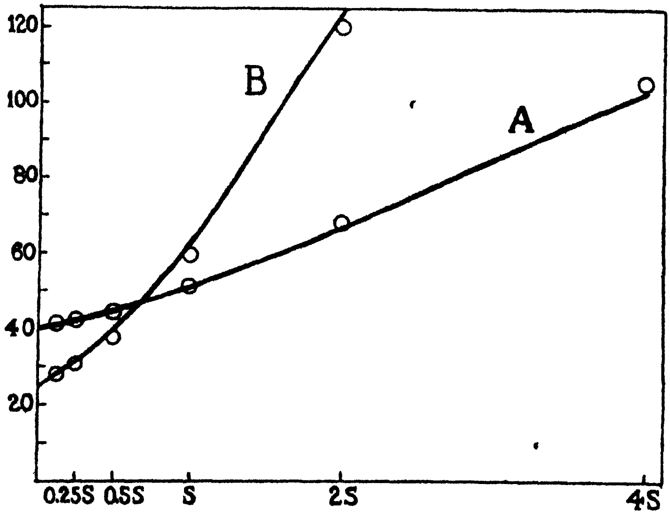

FIG. 2.—Curves showing the relation of number of cells to asymptotic concentration of lysin. Curve A, rabbit cells. Curve B, sheep cells. The figures on the ordinate apply to curve B, and, when divided by 10, to curve A. For further explanation, see text.

separate; the line in fig. 2 thus really turns off parallel to the n-axis at the point where $n = 2 \times 10^4$, instead of continuing without a break until $n = 0$. Such a suspension, however, is about $0 \cdot 0002 \times$ standard in strength, and so there is no possibility of ascertaining whether this deduction is correct or not.

(ii) If the number of cells in the hæmolytic system is sufficiently large, the sum of the individual zones of action must become equal to the volume of the entire system; if the number of cells is increased above this number, the quantity of lysin apparently used up should increase to exactly the same extent, for there are no longer regions in the hæmolytic system which are unoccupied by reacting lysin molecules. The curve relating c_∞ and n must accordingly show a discontinuity when $n = V/v'$; up to that point it will show the type of relation expressed in (3), but beyond that point a doubling of n will result in a doubling of c_∞.

It would be a simple matter to detect this discontinuity, were it not for the following considerations:—(*a*) When v' is small, the discontinuity does not occur until n is too great for accurate values of c_∞ to be obtained,, *e.g.*, v' for rabbits' cells and saponin is 4646 μ^3, whence the value of n at which the discontinuity should occur is $4 \cdot 3 \times 10^8$ cells, a number corresponding to the

addition of a suspension a little more than 4 × standard. The demonstration of the existence of the discontinuity would accordingly depend on the value of c_∞ obtained for an 8 × standard suspension, but end-points are so difficult to determine with such a suspension that sufficiently reliable readings cannot be obtained. (*b*) When v' is large the discontinuity occurs when n is much smaller, but the resulting change in slope of the curve may be so small that it is difficult to detect. The value of v' for sheep cells and saponin, for example, is 12,120 μ^3, and so the discontinuity accordingly should take place where $n = 1 \cdot 7 \times 10^8$ (a standard suspension, approximately); inspection of the preceding table will show, however, that exactly the same result is obtained for a 2 × standard suspension whether we double the value of c_∞ for the standard suspension or continue to calculate the value for the 2 × standard suspension from expression (3). The value of c_∞ for the 4 × standard suspension, on the other hand, strongly suggests that the discontinuity is present, for the value calculated from (3) is 383 microgrammes; the value observed, however, is approximately twice that observed for the 2 × standard suspension, as it should be if the discontinuity under discussion were present.

(*b*) *Evaluation of the Radius of the Zones.*—The distance ρ to which the zone of action extends outwards from the cell surface (the "radius" of the zone) can be found from the equation

$$\rho = \sqrt[3]{\frac{3\mathrm{V}(\mathrm{R}-1)}{4\pi n}} - r, \tag{6}$$

as was pointed out in one of my earlier papers. Here V, R, and n have the same meanings as above, while r represents the radius of a sphere of the same volume as the cell. This sphere is supposed to occupy the centre of the zone, which extends outwards in all directions for a distance ρ.

In order to obtain accurate values of (R — 1), it is necessary to continue the observations for at least 7 hours, *i.e.*, to take as c_∞ the quantity of lysin which produces complete lysis in this time. Only recently, however, have I recognised the necessity for these prolonged observations, and so the values for ρ given in my earlier papers require revision. This revision almost invariably results in the old value for ρ being replaced by a larger one, for, when two time-dilution curves approach different asymptotes and with different velocity constants (the relation being those contained in expression (5)), values of R calculated from observations lasting only 30 to 60 minutes are considerably less than the values calculated from observations extending over many hours. The values of ρ published in 1927 for hæmolytic systems

containing saponin, were as follows:—rabbit, 4·5 μ; man, 5·8 μ; ox, 9·0 μ; and sheep, 9·5 μ; these may be compared with the values in the first column of the table below, which gives the value of v' and of ρ for these four types of cell in systems containing saponin, sodium taurocholate, and digitonin, and also the value of c_∞ for a suspension of standard strength.

Animal.	Saponin.			Taurocholate.			Digitonin.		
	c_∞.	v'.	ρ.	c_∞.	v'.	ρ.	c_∞.	v'.	ρ.
Rabbit....	5·2	4,646	7·9	182	26,140	15·9	10	8,533	10·2
Man	18	9,600	10·2	220	27,270	15·7	12	10,200	10·4
Sheep	60	12,120	12·2	330	23,000	15·6	10	8,533	10·7
Ox	80	13,800	12·7	200	23,400	15·3	12	10,200	11·1

These observations, and, indeed, all others which I have made during the last few years, confirm the conclusion that the radius of the zone of action increases as the activity of the lysin becomes less, or as the resistance of the cell becomes greater. The volume of the individual zone, for example, is approximately the same for systems containing saponin and for systems containing digitonin, two lysins comparable in activity, while it is very much larger for systems containing sodium taurocholate, a much weaker lysin; so far as any one lysin is concerned, moreover, it is generally the case that the type of cell which presents the greater resistance is associated also with the larger zone of action. The relation between resistance and the size of the zone is not, however, as perfect as I have indicated in a previous paper (2), for it is only approximately true, even in the case of any one lysin, that the volume of the zone is proportional to the resistance offered by a suspension containing a constant number of cells. If the volume of the zones in systems which contain different lysins are compared, moreover, the linear relation between their volume and the resistance of the cell becomes even more inadequate. There is no obvious and necessary relation, on theoretical grounds, between the volume of the zone and the resistance of the cell which it is supposed to surround, and so, since no empirical relation is found as a result of experiment, the point may be left undecided and a subject for future investigation.

(c) *The Relation between the Constants of Time-dilution Curves.*—The simplest way of testing the relations contained in expression (5) is to obtain two or more time-dilution curves for the same lysin acting on different numbers of cells, and then to express in each case the times required for complete lysis by dilutions 10 per cent., 20 per cent., ..., etc., of the asymptotic dilution.

It has been shown in the paper immediately preceding this that, when the results are expressed in this way, and when both time-dilution curves have the same value of p, the ratio of the two times corresponding to any percentage value of the asymptotic dilution δ_∞ is constant, and is the same as the ratio of the velocity-constants of the two curves. Further, in the case under consideration, the ratio of the two velocity-constants should be the same as that between the asymptotic concentrations for the two curves.

This method of dealing with the results is best illustrated by an example. Three time-dilution curves were plotted for saponin acting at 22° on (i) a $4 \times$ standard suspension of human cells, (ii) a standard suspension, and (iii) a $0 \cdot 25 \times$ standard suspension. The three asymptotic dilutions were 1 in 80,000, 1 in 110,000, and 1 in 160,000, respectively. The time-dilution curves were plotted in the usual way, and from them were read off the times corresponding to 20 per cent., 40 per cent., ..., etc., of the respective asymptotic dilutions. These times are shown in the following table.

Dilution per cent. of δ_∞.	0·25 standard.		standard t, observed.	4 × standard.	
	t, observed.	t, calculated.		t, observed.	t, calculated.
20	2·0	1·9	1·3	1·0	0·95
40	9·0	8·7	6·0	3·8	4·4
60	40·0	36·4	25·0	17·0	18·2
80	130·0	116·3	80·0	60·0	58·0

Let us denote the constants of the first curve ($0 \cdot 25 \times$ standard suspension) by x_1, p_1, and k_1, those of the curve for the standard suspension by x_2, p_2, and k_2, and those for the curve for the $4 \times$ standard suspension by x_3, p_3, and k_3. Then $x_2/x_1 = 1 \cdot 454$, and $x_3/x_2 = 1 \cdot 375$; we accordingly multiply the values of t for the standard suspension by $1 \cdot 454$ in order to get the calculated values of t for the $0 \cdot 25 \times$ standard suspension, and divide the same values by $1 \cdot 375$ in order to get the calculated values of t for the $4 \times$ standard suspension. The correspondence between the observed and the calculated values given in the above table at once indicates that the expected relations between k_1, k_2 and k_3 exist in fact, and at the same time proves that $p_1 = p_2 = p_3$.

These relations are always easily demonstrated with saponin and rabbit or human cells, for which the zone of action is comparatively small, and also with hæmolytic systems containing sodium taurocholate. I have not obtained such satisfactory results, however, with saponin and sheep or ox cells, the times observed for the $4 \times$ standard suspension being usually longer than those

calculated. I attribute this to the difficulty in obtaining good end-points with such concentrated suspensions, for, when weaker suspensions are used, even these hæmolytic systems give results as expected from expression (5).

Finally, I should again emphasise a point which was mentioned when this hypothesis of the local action of lysin molecules was first described. Although excellent correspondence exists between the observations of the experiment and the predictions made on this particular theory, and although this correspondence can be taken to indicate that the primary assumption, *i.e.*, the existence of a localised action, is correct, it cannot be taken as proof that all the assumptions made are correct in detail. The hypothesis differs, in fact, from all others introduced in connection with the kinetics of the simple hæmolysins, in that it can be verified by one type of experiment only, and only in a very indirect way; and it is quite possible that, from rather different initial assumptions, expressions similar to, or even the same as, those which have been shown above to describe the experimental results could be set up. It is, for example, quite probable that the zone is not sharply defined, and that it may vary in volume at various stages of the reaction and even with varying lysin concentration; as remarked in a previous paper, however, the accordance of the experimental results with those predicted, as well as the reasonableness of the conclusions to which the conception leads, permits us to regard the hypothesis as satisfactory in its essentials and as useful for descriptive purposes at least.

Summary.

On the basis that simple hæmolysins act only at the surfaces of the cells of hæmolytic systems, equations which describe the effect of varying the number of such cells are developed in this paper. The resulting expressions are in general similar to those published in 1927; in this investigation, however, their validity is tested over a much greater experimental range and for hæmolytic systems containing saponin, sodium taurocholate, and digitonin together with the cells of the ox, the sheep, the rabbit, and man.

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The Form of the Frequency Distribution of Red Cell Resistances to Saponin.

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Although nearly all investigations on the kinetics of hæmolytic reactions have assumed some kind of chemical reaction taking place in a population of cells which offer various degrees of resistance to the lysin, the distribution of these individual resistances is usually regarded as something which either has to be postulated, or which is derived indirectly by a particular treatment of experimental data, rather than as something which can be shown to exist by direct experiment. This paper is concerned with the description of a method whereby the frequency distribution of resistances can be arrived at directly, and with a comparison of the results obtained with those obtained by indirect methods.

I. *General Considerations.*

When a simple lysin, such as saponin, acts on a suspension of washed mammalian red cells, it is easy to satisfy one's self that the lysis is accompanied by a using up of saponin, the hæmolysin presumably combining with some component (probably protein) in the cell membrane. This reaction may be called the "fundamental reaction," and its velocity can be expressed by the following equation

$$dx/dt = k\,(c - x)^n\,(\mathrm{S} - x)^m, \tag{1}$$

in which x is the quantity of lysin which unites with the component S, c is the initial quantity of lysin, and k, m, and n are constants. When suspensions of the same number of cells are being considered, it is found in practice that the term containing S can be treated as constant, and the expression then takes its more usual form

$$dx/dt = k\,(c - x)^n. \tag{2}$$

When this is integrated, it provides us with the relation between the time t required for the formation of any given quantity of x, and the initial concentration of lysin c,

$$kt = \frac{p}{p-1}\cdot\left\{c^{\frac{p-1}{p}} - (c - x)^{\frac{p-1}{p}}\right\}. \tag{3}$$

Here n is written as $1/p$, for convenience. The fundamental reaction, accordingly, is of the nth order, and its velocity at any moment dependent on the nth power of the concentration of free lysin present at that moment.

Expression (3) gives the quantity of lysin (usually measured in microgrammes) combined with the cells at the end of any given time, but it does not, as it stands, give the amount of lysis which results from this combination. The reason for this is that the cells offer different resistances to the lysin, some hæmolysing when only a small amount of their component is combined with saponin, and others requiring larger quantities of their component to be transformed before hæmolysis takes place. The resistance of the cell is thus measured in terms of the quantity of lysin, which, by combining with some component of its membrane, hæmolyses it. In a population consisting of a large number of cells, these resistances must be distributed in some particular way, and the most likely forms of distribution are either

$$n = n_0 (1 + x/a_1)^{m_1} (1 - x/a_2)^{m_2}. \tag{4}$$

which is a skew curve of Pearson's type I, or the symmetrical frequency curve

$$n = n_0 e^{-b^2x^2}. \tag{5}$$

In these expressions n denotes the number of cells corresponding to any resistance x, although, since the origin in each case is the mode, the numerical value of corresponding x's in (2) and in (4) or (5) are not the same. This fact is easily allowed for in graphical analysis.

As the fundamental reaction proceeds accordingly to (2), quantities of lysin x_1, x_2, x_3, etc., combine with the cells in times t_1, t_2, t_3, etc. The group of cells whose resistance corresponds to x_1 hæmolyse when x_1 is formed, and their number is found from (4) or (5). When x_2 is formed in time t_2, the group of cells whose resistance corresponds to x_2 hæmolyse, and their number is added to the number already lysed. And so on for all values of x; in general, the number of n cells hæmolysed after any time t from the commencement of the reaction will be given by solving (3) simultaneously with

$$n = n_0 \int_0^x (1 + x/a_1)^{m_1} (1 - x/a_2)^{m_2} \, dx, \tag{6}$$

with

$$n = n_0 \int_0^x e^{-b^2x^2} \, dx, \tag{7}$$

as the case may be. The result in each case is to give a more or less S-shaped "percentage hæmolysis curve."

There are several methods of obtaining these S-shaped percentage hæmolysis

curves experimentally, but it will be clear from the foregoing that it is impossible to obtain the characteristics of the frequency distribution of resistances from such curves alone. Put briefly, the reason for this is that the percentage hæmolysis curve gives the number of cells hæmolysed at various intervals of time, whereas the resistance distribution expresses the number of cells which hæmolyse as different quantities of x are formed ; to deduce the latter relation from the former is obviously impossible unless we have a third relation, *i.e.*, that between time and the formation of various quantities of x. There are accordingly only two ways of finding the form of the frequency distribution. In the first a percentage hæmolysis curve is obtained, the constants of (3) are found from a time-dilution curve, and that form of distribution is calculated which, together with a fundamental reaction described by (3) with the particular value of the constants inserted, will give the experimental percentage hæmolysis curve. In the second, expression (2) is eliminated altogether by using the special condition that where $t = \infty$, $x = c$ and an approximation to the frequency distribution is obtained directly.

Before considering the form of the distribution in detail, it should be observed that expression (3) can take at least two special forms :—

(i) If $n = 1$, the expression becomes one of the first order, or "monomolecular"

$$kt = \log \frac{c}{c - x}. \tag{8}$$

(ii) If c is very great compared to x, expressions (2) and (3) tend to become

$$dx/dt = \text{const.}, \quad \text{and} \quad t = kx/c. \tag{9}$$

These two cases will be seen to be of considerable importance in the discussion following.

II. *The Form of the Frequency Distribution of Resistances to Saponin.*

As has been already stated, there are two methods of finding the frequency distribution of the resistances of red cells to a lysin. The first, which may be termed the indirect method, has been already fully described, and depends on calculations based on experimental time-dilution and percentage hæmolysis curves (Ponder, 1926, 1927 ; Ponder and Yeager, 1930). The second method is simple and direct, and will be described below.

Method.—The only apparatus required is a water bath at a convenient temperature, a number of clean dry test-tubes, 100 × 13 mm., a 1-c.c. pipette graduated in 1/100ths, a series of dilutions of saponin in 0·85 per cent. NaCl, a

quantity of a suspension of washed red cells, and a colorimeter capable of working with volumes of about 1 c.c. The red cell suspension is made by suspending the thrice washed cells of 1 c.c. of blood in 20 c.c. of 0·85 per cent. NaCl, and should be freshly prepared.* The dilutions of saponin required (for human cells) are :—1 in 80,000, 1 in 90,000, 1 in 100,000, 1 in 120,000, 1 in 140,000, 1 in 160,000, 1 in 180,000, 1 in 200,000, 1 in 240,000, 1 in 300,000, 1 in 360,000, 1 in 400,000, 1 in 500,000, 1 in 600,000, 1 in 800,000, and 1 in 1,000,000, but each should contain 2·5 times as much lysin as the dilution figure indicates, for a reason to be seen below. These dilutions are kept in stock bottles (100 c.c.), and are preserved in a refrigerator.

Of each dilution of lysin 0·8 c.c. is taken and placed in a tube ; 0·8 c.c. of 0·85 c.c. NaCl is then added, and the mixture shaken. To the mixture 0·4 c.c. of cell suspension is added, thus diluting the lysin initially added 2·5 times, and making the final dilution in the tube the same as that marked on the stock bottle. The tubes are kept in the water bath at constant temperature for 7 to 10 hours, and every 15 minutes or so they are shaken. This regular shaking is very important. At the end of the time each tube is centrifuged to throw down the unhæmolysed cells, and the supernatant hæmoglobin-stained fluid is matched against standards representing 100 per cent., 75 per cent., 50 per cent., and 25 per cent. and 10 per cent. hæmolysis. The accuracy of matching is considerably increased if the colorimeter is fitted with a Newcomer eyepiece.

After a time as long as 7 hours, the velocity of the fundamental reaction between cells and lysin is very small, and the quantity of lysin transformed, x tends to be equal to (or, more accurately, proportional to) c, the quantity of lysin initially present. Thus, if the amount of hæmolysis in each tube, expressed as a percentage of complete lysis, is plotted against the initial quantity of lysin in each tube, expressed in milligrammes or microgrammes, the result is a curve which is a fairly good approximation to the integral of the frequency distribution of red cell resistances to the action of the hæmolysin.

Results.

It is convenient to discuss the results under several heads : (*a*) the range of the distribution, (*b*) its form, (*c*) its constancy for the cells of any individual, and (*d*) the accuracy with which the distribution may be determined.

* The cells, of course, may be suspended in a variety of physiological saline solutions, in which case the same saline is used for dissolving the lysin and also for completing the 2 c.c. volume of the system. It cannot be too strongly emphasised that different results may be, and usually are, obtained when the suspension medium is altered.

(*a*) The upper extreme of the distribution is remarkably constant in position, the most usual value at 22°, for a standard suspension of human red cells, being 20 microgrammes (corresponding to a lysin dilution of 1 in 100,000). This constancy confirms previous observations both of my own (Ponder, 1926) and of Bodansky (1928). The lower limit is not nearly so constant, for it may vary from 2 microgrammes to 6 microgrammes, even for the cells of the same individual. The most resistant cells are thus from about three times to about ten times as resistant as the least resistant, but the variation is principally due to the inconstancy of the resistance of the latter group.

Since there are 10^8 cells per tube, and since the quantity of lysin used up in bringing about their hæmolysis is of the order of 10 microgrammes at the very most, it follows that 10^{-13} grammes of lysin is the maximum quantity available as entering into combination with each cell. This is quite a small quantity relative to the mass of the membrane, which is probably of the order of 10^{-12} grammes as a minimum. The amount of lysin utilised is so small indeed as to invite speculation as to whether it could even form a monolayer. Too little is known regarding the saponin molecule for the calculation to be made, but it can be made for sodium oleate, a lysin of nearly identical activity. The molecular weight is about 300 ; 10 microgrammes thus contain about 2×10^{16} molecules, whence the number of molecules available for each cell is 2×10^8. The dimensions of the sodium oleate molecule, according to Du Nouy, are such that the greatest area it can cover is about $84 \times 10^{-8}\ \mu^2$; the molecules available for each cell would accordingly cover about $170\ \mu^2$ if arranged in a monolayer, *i.e.*, an area just about equal to that of the cell surface. This figure, however, is a maximum estimate, for there is evidence that all the lysin molecules in the tube do not react with the cells (Ponder, 1926) ; it is therefore quite probable that lysis can occur even when there are too few lysin molecules to form a monolayer at the cell surfaces.

(*b*) The most remarkable feature of the frequency distributions is their extreme variability in form, some curves being so negatively skew as to be almost J-shaped, while others are so moderately skew, that, when seen in their integral form, they appear almost symmetrical.* Although much more investigation is required before these distributions can be classified according

* When speaking of these integral curves, the terms " moderately skew," and " virtually symmetrical " are not used in the exact sense in which a statistician would employ them. I recognise, for example, that even the most symmetrical of the curves obtained is by no means truly so, and also that some " moderately skew " curves have a skewness of about $-0 \cdot 5$.

to type and frequency, two conspicuous varieties can be recognised in the meantime :—(i) a very skew curve with a long "tail" (fig. 1, A), and (ii) a very flat curve with a smaller degree of skewness, but also with a considerable "tail" at its upper end (fig. 1, B). Between these two types all intermediate

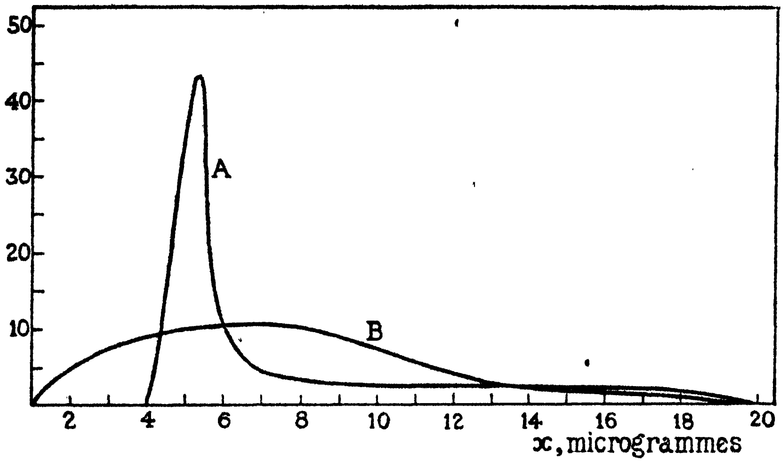

Fig. 1.—Two observed frequency distributions, human red cells and saponin.

forms occur. Curves more skew than (i), and also curves more nearly symmetrical than (ii), occur with the cells of animals other than man, and so there appears at the present time to be little restriction on the forms of distributions met with, except that positively skew curves can be excluded.

(*c*) The great variety of types of frequency distribution met with is contributed to by the fact that the type of distribution is not constant for the cells of the same individual and for the same lysin, for it is apparently affected by minor factors which are difficult to control. The distribution is different, for example, when the cells are washed in 0·85 per cent. NaCl from what it is if they are washed in isotonic citrate, and differs according as to whether the dilutions of lysin used to obtain it are freshly prepared or not. This variability makes it difficult to compare results from day to day; indeed, it is quite impossible to compare results at all unless the technique is standardised down to the smallest detail.

(*d*) All the above results must be interpreted in relation to the accuracy of the method used, and this is subject to several sources of error which require to be mentioned. (i) The method is based on the fact that, irrespective of the order of the reaction, $x = c$ when $t = \infty$. In practice t is only from 400 to 500 minutes, and the value of x for each tube is accordingly not equal to the value of c. The discrepancy introduced in this way depends on the order of

the reaction, and can be calculated exactly only when the latter is known; for approximate purposes, however, and when distributions covering the same range as those considered in this paper are concerned, the true value of x may be taken as more nearly equal to $(c - 1)$ than to c itself. The making of this correction accordingly moves the entire distribution 1 unit in the direction of the origin. (ii) The colour matching, by which the number of cells hæmolysed is determined, is associated with quite considerable errors when this number is small; the lower end of the distribution is accordingly likely to be none too correctly mapped out. (iii) It must always be borne in mind that the frequency distributions are obtained in their integral form, whether by the direct method or by any other procedure, and that such integral curves are frequently very misleading. It is a well-known fact, for example, that two very different frequency distributions may yield integrals which look quite similar to the eye, and that what looks like a remarkably good correspondence between a number of experimental points and a sigmoid curve may be really a very poor correspondence indeed. It is therefore necessary to be on one's guard against being misled by spurious accuracy, and to remember that the drawing of sigmoid curves inevitably results in a certain amount of "rounding off" of the data, to an extent, indeed, which is sometimes surprising.

III. *Comparison with Existing Observations.*

In comparing the results obtained by this direct method with those obtained by myself and other observers by other methods, it is necessary to bear in mind that the theory outlined above has been arrived at over a number of years, and by the process of replacing the first approximations which quite adequately described the earlier experimental finding by more elaborate expressions demanded by the results of more extensive and perfect investigation. As is usual in such cases, the slow and somewhat halting development of the theory has been due far more to the inadequacy of experiment than to any real theoretical difficulty, with the result that several quite erroneous statements of the true state of affairs have seemed fully supported by the evidence at the time.

In the development of the present theory there have been at least four stages, the first of which is marked by the introduction, by Arrhenius (1914), of the idea that lysis is a chemical reaction. It was previously looked upon as a process of solution of lipoids of the red cell membrane, an idea which has never had any evidence in its favour. Arrhenius, however, seems neither to have defined "resistance" nor to have taken into account the fact that lysin,

as well as intact cells, disappears during the reaction, for he wrote the velocity of the process as

$$dn/dt = k\,(N_0 - n) \tag{10}$$

in which n denotes the number of cells destroyed, and N_0 the initial number. This expression really assumes the frequency distribution of red cell resistances to be a rectangle, for it becomes compatible with (2) and (6) if $dn/dx =$ constant; the implication, however, was not realised at the time, and the investigations of Arrhenius, his pupils, and their opponents were principally concerned with the applicability of (10) to percentage hæmolysis curves. Much of the controversy turned on whether the typical percentage hæmolysis curve is S-shaped, or uniformly concave to the time axis as expression (10) would demand, and, although the point was difficult to decide owing to the methods of measuring percentage being inadequate, the consensus of opinion by the year 1920 seems to have been that percentage hæmolysis curves are sigmoid.

In 1923 I introduced the radiometer method for the measurement of percentage hæmolysis (Ponder, 1923) and obtained sigmoid curves consistently, but because of the peculiarities of the particular radiometer apparatus used, I worked at 12° and with a red cell suspension about one-quarter the strength usually employed. More unfortunate conditions could scarcely have been selected, for at least three reasons: (i) At low temperatures the velocity constant of the hæmolytic reaction is large, and even when the initial concentration of lysin is great the time required for complete lysis is considerable, *e.g.*, when $c = 40$ microgrammes, $t = 27$ minutes. (ii) When the cell suspension is dilute x tends to be small; combining this with (i), it will be clear that the conditions employed were such that c was unusually great compared with x, *i.e.*, such that $(c - x)$ tended to be constant, even when quite protracted reactions were under consideration. Under such circumstances expression (9) may describe the results quite well, for the velocity of the fundamental reaction tends to be constant, and such a reaction, proceeding in a system, containing cells whose resistances are symmetrically distributed, give symmetrical sigmoid percentage hæmolysis curves. Indeed, since $dx/dt =$ const. the percentage hæmolysis curve, whose axes are n, a number of cells, and t, the time required for the lysis of any number, is itself the integral (except for a multiplying constant) of the frequency distribution, whose axes are n and x, a quantity of lysin transformed. (iii) As has already been pointed out, most frequency distributions of resistances show a long "tail" in the direction of the upper extreme. With a dilute suspension in which lysis is being measured by the radiometer it is easy to think lysis complete before 100 per cent. lysis is

reached; this error tends to abolish the "tail," and with it much of the skewness of the distribution.

When the radiometer method gave virtually symmetrical sigmoid percentage hæmolysis curves, I concluded that the cell resistances are ideally distributed, and that the reaction between cells and lysin is constant in velocity. Even at the time, however, I remarked that the second conclusion is a curious one; knowing nothing of the nature of the fundamental reaction, I accounted for it on the grounds that the fundamental reaction continues as stromatolysis long after complete lysis of the cells, and that a short part of any such prolonged reaction can be regarded as having constant velocity.* The first conclusion, on the other hand, seemed so inherently likely that even when skew percentage hæmolysis curves were obtained I believed that they could be accounted for by assuming that liberated substances, such as hæmoglobin, retard or accelerate the fundamental reaction, the frequency distribution, however, still being assumed to be symmetrical.†

Further work with the radiometer, together with investigations in which the selenium cell was used for measuring percentage hæmolysis (Ponder, 1926, 1927), soon showed, however, that the form of percentage hæmolysis curves varies as the initial concentration of lysin is varied, the curves obtained when relatively high concentrations of lysin are used being more symmetrical, for example, than those obtained when the lysin is dilute; percentage hæmolysis curves alone, accordingly, tell us little about the resistance distribution, for, if we regard the former as integrals of the latter, we get as many new distributions as the concentrations of lysin we employ. Further, most of the percentage hæmolysis curves obtained were negatively skew, relatively symmetrical curves being found only at low temperatures or with concentrated lysins. This fact, together with a study of time-dilution curves, led to the hypothesis, put forward in 1926, that the fundamental reaction is of the first order and described by expression (8), while, as before, the frequency

* This is actually the case, and it is partly for this reason that the term $(S - x)$ in expression (1) can be regarded as constant; were this not so, the fundamental reaction would have to be treated as a "bimolecular" type—which, indeed, may ultimately turn out to be necessary. In the meantime, however, it seems that the component S is greatly in excess of the amount of lysin transformed at the stage of complete lysis. Under any circumstances, it would be very difficult indeed to establish that a "bimolecular" reaction is preferable to a reaction of a simpler form.

† We now believe that these liberated substances affect, not the velocity of the funda mental reaction, but the distribution of resistances (Ponder, 1926).

distribution of resistance is symmetrical, and described by (7) or by an expression of a similar type.

The combination of these two expressions gives negatively skew percentage hæmolysis curves whose skewness decreases as the concentration of lysin increases; excellent agreement was obtained between calculated and experimental results, and the hypothesis accordingly seemed satisfactory, although it had to be modified in one respect. Frequently the skewness of the percentage hæmolysis curves was found to be greater than calculated on the basis of a symmetrical distribution; it then became necessary to postulate a distribution which itself showed negative skewness (Ponder, 1927; Ponder and Yeager, 1930).

Finally, for reasons already fully discussed (Ponder and Yeager, 1930), it has been found necessary to regard the fundamental reaction as one of the nth order, the value of n usually being, when saponin is used as a lysin, about 2·0. Expression (3) thus replaces (8), just as (8) previously replaced (9), and the percentage hæmolysis curves are given by solving expression (3) simultaneously with the proper expression for the frequency distribution.

The question raised in this paper is that of the nature of this latter expression, and the direct method of investigation adopted has shown that the distribution may vary from one which is so negatively skew as to be nearly J-shaped, to one which, were it not for a long "tail," would be virtually symmetrical. The results of the direct method, however, may be compared with the results obtained by indirect methods, there being four sets of data in particular which must be considered: (*a*) my own recent observations with the potassium cell, (*b*) the observations of Kesten and Zucker (1928), by a similar method, (*c*) the observations of Kesten (1929), by a method in which the resistance of single cells is observed, and (*d*) the observations of Orahovats (1926).

(*a*) A typical set of percentage hæmolysis curve for various dilutions of saponin is shown in fig. 2, the percentage number of cells hæmolysed being observed at various times by the potassium cell method. As explained above, only one distribution of resistances, described by an expression of the type of (6), need be postulated to account for all these curves, for the differences between them can be accounted for by differences in the rate of the fundamental reaction described by expression (3). In this particular case, $n = 1 \cdot 8$ and $k = 0 \cdot 0059$, these values being found from a time-dilution curve for the same hæmolytic system; the subsequent graphical analysis then supplies us with the integral of the frequency distribution of resistances, shown in fig. 3, which integral is the same irrespective of the particular percentage hæmolysis curve

from which it is derived. The derivative of this curve, which represents the actual distribution, is seen in the same figure.

As can be seen from the figure, this distribution has essentially the same characteristics as some of those obtained by the direct method. There is a

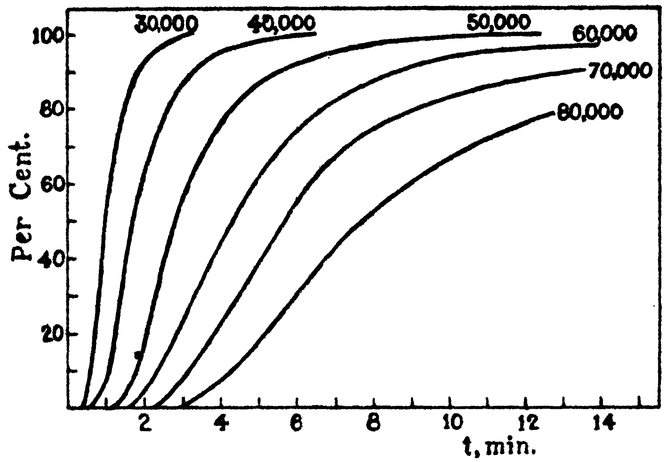

FIG. 2.—Set of percentage hæmolysis curves at 25°, human red cells and saponin. The figures near each curve indicate the dilution of lysin used.

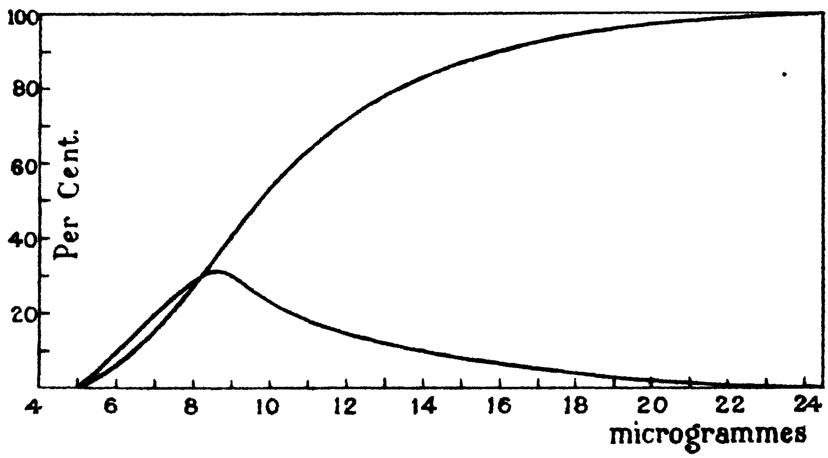

FIG. 3.—Frequency distribution for human red cells and saponin together with its integral. Obtained by calculation from the curves shown in fig. 2.

moderate degree of negative skewness, and the usual " tail " in the direction of the upper limit. This particular distribution, however, is only an example of the general type of result obtained from the analysis of percentage hæmolysis curves, for as considerable differences in the form of the distribution are

observed by this method as by any other. Some distributions are more symmetrical and some are more negatively skew; probably the particular form obtained is dependent on many factors relating to the physical state of the lysin and the condition of the cells.

Both the direct method described above and the method of analysing percentage hæmolysis curves accordingly agree as to the most common form of distribution being one which is negatively skew. The question now arises as to whether the occurrence of symmetrical or even of positively skew distributions can be excluded. I think that it cannot be excluded, at least without much further investigation. It is significant, however, that as methods of investigation become more accurate, symmetrical curves become more and more rarely described; this, I think, can be accounted for on two grounds: (i) if percentage hæmolysis curves are analysed on the supposition that the fundamental reaction is one of the first order when it is really of a higher order, the negative skewness of the distribution is partly disguised; it is only recently that the true order of the fundamental reaction has been recognised, and so much of the symmetry found in earlier investigations may be spurious; (ii) owing to the long "tail" possessed by so many distributions, their skewness is apt to be underestimated if the time for complete hæmolysis is underestimated, or, more precisely, if 97 to 99 per cent. lysis is considered as "complete." This error is easy to make, especially by the older experimental methods (*e.g.*, the radiometer method, which is quite insensitive in the region of 100 per cent. lysis), and it is quite possible that it has contributed to the symmetry of some of the distributions described in earlier investigations. It should be observed, however, that the question of the symmetry or asymmetry of these distributions has nothing to do with the criticism made by Kesten and Zucker, that they find the usual percentage hæmolysis curve to slope off gradually above 90 per cent. hæmolysis, whereas I have described it as rising promptly to 100 per cent. It is easy to see from the foregoing that the promptness with which such a curve rises to the point of complete lysis depends principally on the concentration of lysin used in the experiment, and that two sets of percentage hæmolysis curves, obtained with different concentrations of lysin, different concentrations of cell suspension, and, at different temperatures, cannot be compared unless the differences are allowed for. By using the conditions under which Kesten and Zucker worked I can reproduce their results, just as by using the conditions under which I have worked they could no doubt reproduce mine. Indeed, I shall show below that the two sets of results are mutually confirmatory.

(*b*) Recently Kesten and Zucker (1928), on the basis of a series of studies of percentage hæmolysis curves by the potassium cell method, have been led to "seriously question the importance attributed by some writers to the probability distribution." Their principal reasons for this conclusion appear to be that the distributions found by them are more skew than they expect, and they think that the hæmolytic reaction must be influenced by the constantly decreasing cell concentration as lysis proceeds.* Both of these investigators fail to realise that the frequency distribution of resistances is one in which a number of cells is a function of a quantity of lysin used, and not a function of time; they omit, indeed, any reference to a reaction between cells and lysin and in consequence they reach conclusions which are clearly contradicted by their own results. I shall accordingly take some of these results at random and show that, if properly analysed, they lead to conclusions identical with those reached in this paper.

In fig. 4, A, is shown a representative percentage hæmolysis curve taken from one of Kesten and Zucker's papers. Its derivative is not shown, but,

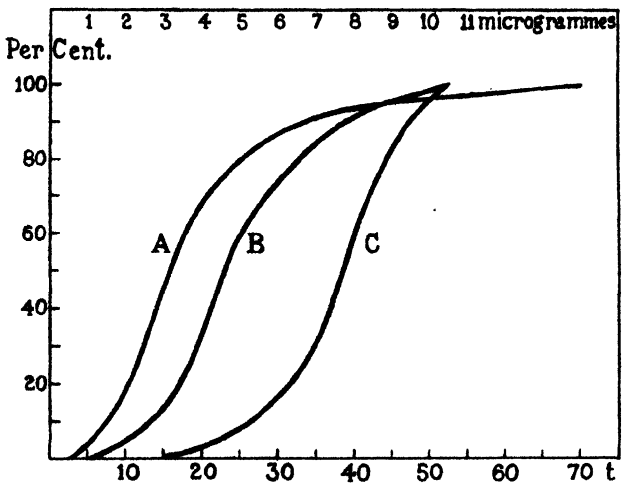

Fig. 4.—Integrals of frequency distributions calculated from Kesten and Zucker's percentage hæmolysis curve A. Curve B shows the integral of the distribution on the assumption that $n = 1$; curve C on the assumption that $n = 2$. The lower scale refers to curve A, upper scale to curves B and C.

* It is not, however, the "concentration of cells," in the sense of the number of intact cells, upon which the velocity of the hæmolytic reaction depends, but rather on the concentration of the cell component with which the lysin reacts. There may be plenty of this component, continuing to react with the lysin, even after a cell is hæmolysed; this continued reaction, indeed, results in the breaking up of the "ghosts."

as can be seen in the original figure, it is a frequency distribution of type I, with a considerable negative skewness. This distribution is that of the cell resistances only when dx/dt = const. There is no way of discovering the order of the fundamental reaction in this particular case, for data are given for one concentration of lysin only; the order, however, is certainly not zero, and I shall assume first that it is 1·0, and then that it is 2·0. I shall also assume, in absence of data, that the asymptote of a time-dilution curve for Kesten and Zucker's system would be 10·53 microgrammes,* for the concentration of lysin used was 1 in 167,000, and this produced complete lysis in 50 to 70 minutes. Analysing the percentage hæmolysis curve by means of expression (3), curve B of fig. 4 is found for the integral of the resistance distribution if $n = 1 \cdot 0$, and curve C if $n = 2 \cdot 0$, the scale to be used in the case of each being given above the curves, and its units being units of lysin transformed.

The distribution whose integral is curve B is very similar to those described earlier in this paper; indeed, it is more symmetrical than most. That corresponding to curve C is actually positively skew. The "distribution" corresponding to curve A, on the other hand, means nothing at all, and could be rendered either more or less skew by changing the concentration of lysin used.

A similar analysis applied to any of the percentage hæmolysis curves published by Kesten and Zucker will lead to the same conclusion, *i.e.*, that the distribution of red cell resistances to saponin is substantially the same as those shown in fig. 1. If there is any difference worthy of remark, it is that Kesten and Zucker's distributions are more symmetrical than these latter, but it is impossible to compare the results in detail, for the former were obtained with suspensions which had stood at 2° to 4° for 18 to 20 hours, while the latter were obtained with fresh suspensions. Further, some curves are for the cells of man, and some for rabbits' cells.

(*c*) Within the last few months Kesten has introduced a new method of obtaining what he believes to be curves for the distribution of red cell resistances

* I have selected these values for the constant on the basis of my experience with saponis obtained from the same source as that used by Kesten and Zucker (Eimer & Amend, New York City). I do not, of course, claim that these values are the same as Kesten and Zucker would have found by experiment for their particular hæmolytic system, but, to compensate for the uncertainty, I have endeavoured to choose permissible values which tend to make the resistance distribution as negatively skew as possible, *i.e.*, to support Kesten and Zucker's conclusion. Whatever values, however (within certain obvious limits), were to be assigned to the constants, the result of the analysis would be much the same. These remarks also apply to my analysis of Kesten's curves (see (*c*) above).

(Kesten, 1929). The method consists in observing individual erythrocytes in a solution of lysin, and noting the numbers which hæmolyse in 1, 2, 3, ... 30 minutes. The results, when expressed as a percentage of the total, give, of course, a percentage hæmolysis curve. Kesten claims that this method is more likely to reveal individual differences in cell resistance than the methods commonly in use, but nevertheless that the curves are not sigmoid, but uniformly concave to the time axis* ; further, he thinks that the " distributions " obtained by differentiating these curves are so skew as to raise doubts as to their actual existence, *i.e.*, as to whether the whole subject could not be treated without emphasising the resistance differences.

The first claim is, of course, entirely wrong, for the observation of a limited number of cells makes it less likely, instead of more likely, that a curve will be obtained which represents the true percentage of cells of any one resistance ; as can be seen from the experimental " distributions," the method is not nearly accurate enough to supply a good graduation. A close inspection of the figures in the paper, moreover, shows the second claim to be unfounded also, for several of the curves are distinctly sigmoid. Finally, enough has already been said to dispose of the third claim as well, for the high degree of skewness which Kesten thinks improbable is the result of the same faulty analysis as is used by himself and Zucker, viz., the assumption that the frequency curve for red cell resistance is the derivative of the percentage hæmolysis curve. This point will be best illustrated by an analysis of one of the curves from fig. 4 of Kesten's paper.

The dilution of lysin is 1 in 35,000 and complete lysis occurs in about 60 minutes. I assign the value of $2 \cdot 0$ to n in expression (3), the value 45 microgrammes to x and the value $0 \cdot 001097$ to k. The value for the number of cells hæmolysed at various times I have taken from Kesten's curve B, and the frequency distribution which results is shown in fig. 5, the points being marked by crosses. It has quite a small skewness. To show that this curve is obtained not for the dilution 1 in 35,000 only, but when other dilutions of lysin are used and the same analysis is applied, I have derived the integral also from curve C of Kesten's fig. 4 and, by solving simultaneously with (3) with the same values of the constant inserted, have obtained the points marked in fig. 5 with squares. The agreement is sufficiently good to show that the analysis of the data

* This would not provide evidence against the existence of a frequency distribution even if the observation were correct. It would merely prove the distribution to be J-shaped.

presented in either curve B or C leads to the same result, and that this result is very similar to those obtained by other methods.*

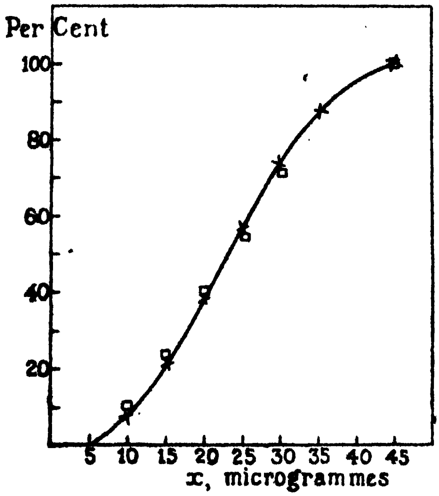

FIG. 5.—Integral of frequency distribution calculated from Kesten's data.

(*d*) Orahovats employed, in 1926, a method very similar to the direct method described above, with the object of discovering if cells from the splenic pulp have a resistance to saponin greater than that possessed by the cells of the general circulation. The resistance integrals obtained in his experiments, although quite adequate for his own investigation, are unfortunately rather inadequate for the present purpose, the time during which the saponin was allowed to act on the cells being only 1 hour; two such distributions, however, are shown in fig. 6, and it is clear that both the curve for the blood of the general circulation and that for the cells of the splenic pulp correspond in their general properties to the integral curves described earlier in this paper. The former gives rise to a flat distribution, moderately skew, and having a long "tail," (curve A, fig. 6), while the latter gives a distribution much more skew (curve B, fig. 6).

The comparison of these four sets of results accordingly makes it quite clear that all observations point to the existence of a frequency distribution of red cell resistances to saponin, and that they further agree with respect to the general forms assumed by this distribution. Very similar results, it may be added, are obtained if other hæmolysins than saponin are used.

* I cannot derive the same distribution from curve A of the same figure. There are, of course, many possible explanations for this.

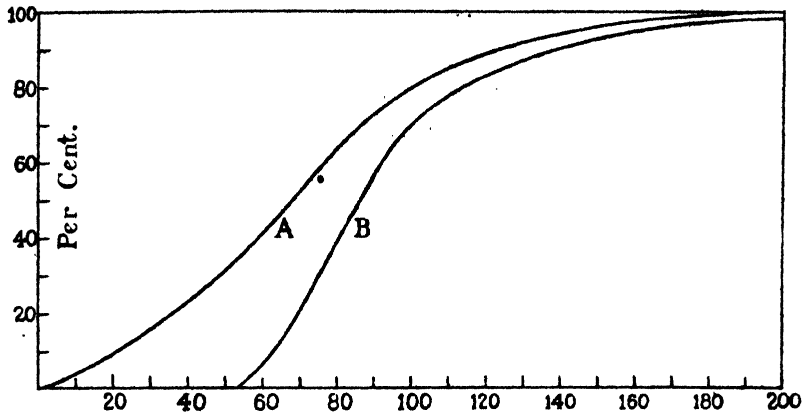

FIG. 6.—Two integrals of frequency distributions calculated from the data of Orahovats.

Summary.

A simple method is described whereby the resistances of red cells to a lysin such as saponin can be found without involved calculation.

The distributions of red cell resistance obtained present a variety of forms, some being moderately skew, and some being almost J-shaped. Considerable variation is met with if the cells of different animals are used, and even with the cells of the same animal at different times. Most distributions, however, are characterised by a long "tail" in the direction of the upper extreme, to the presence of which much of the skewness of the integrated form of the curve is due.

The results obtained by this method are shown to be in agreement with those given by other methods, and it is shown that similar results can be obtained from the data of several other observers.

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Evaporation from the Meal-Worm (Tenebrio : Coleoptera) *and Atmospheric Humidity.*

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Introductory.

It is a matter of general agreement among entomologists that the seasonal and geographical distribution of insects is as much determined by atmospheric moisture as it is by temperature, but it is curious that very little critical work has been carried out on the precise physiology of this problem. As it appears to me, the kernel of the matter is this—it is possible that the relation between the insect and the humidity of the atmosphere in which it lives is "simple," in the sense that the loss of water from the insect depends on some physical law. For instance, it may be that the loss of water is directly determined by relative humidity or by vapour pressure, but it is more probable that it is determined by saturation deficiency ; in saying this I argue from the analogy of the green leaf and the warm-blooded mammal, and also from the physical laws of evaporation. But it is more probable that the relation is "complex" ; for we are not considering the loss of water from a simple or smooth surface, and some at least of the loss will take place within the tracheal system, the tubes of which ramify to all parts of the insect's body: and we may surely feel certain that the air in the tracheal tubes will be more nearly saturated with moisture than that outside, if the insect is kept in a dry atmosphere. We must also remember the possibility that the insect can control loss of water from the tracheal system by closing the spiracles through which the tracheal tubes communicate with the outside air. Furthermore, the general metabolism of the insect may be affected by the moisture or dryness of the atmosphere in which it is living.

In considering our problem it is essential to grasp the differences between the various measures of atmospheric moisture which may be employed. The differences may be appreciated from a study of fig. 1, which sets out the relation between temperature on the horizontal scale and vapour pressure on the vertical. The vapour pressures sufficient to saturate air at various temperatures lie along the curve AB, which is the curve of "saturation vapour pressure."

The straight base line of the graph represents the condition in which air is completely dry. Turning to the other notation, AB corresponds to a relative humidity of 100 per cent., the base line to a relative humidity of 0 per cent. ;

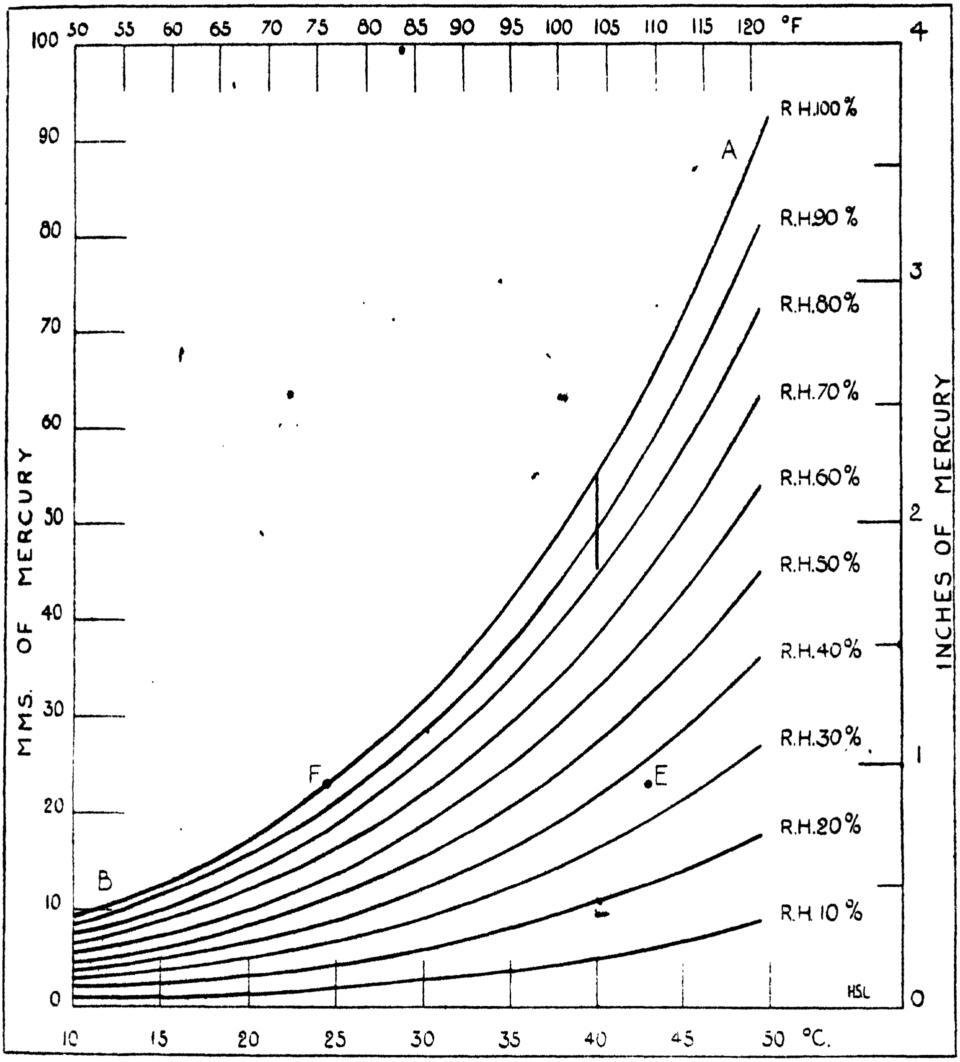

FIG. 1.—Graph for converting readings of relative humidity into vapour pressure or saturation deficiency, and *vice versa*.

the curves for other relative humidities lie between these two, and they are not parallel to either. If now we take a saturation deficiency of 10 mm. at a temperature of 40° C., as indicated by the vertical line on the graph, we arrive at a relative humidity of 82 per cent. ; but the same saturation deficiency at

30° or at 20° corresponds to quite different values of relative humidity. It will be appreciated from this that relative humidity and saturation deficiency are entirely distinct, and that the two notations cannot be converted into one another, unless the temperature is known.* If we could show that the loss of water from an insect was determined by saturation deficiency, we should be in a position to explore the biology and the seasonal distribution of insects in so far as it is determined by the humidity of the atmosphere.

Preliminary Studies and Methods.

In endeavouring to study this question, my general method has been to make atmospheres of a known relative humidity† by putting appropriate quantities of sulphuric acid and water in a desiccator: the desiccators are stored at any desired temperature in an incubator. If I were repeating the work I should prefer to control humidity by appropriate concentrations of potassium hydroxide, for the traces of carbon dioxide which accumulate when larvæ are kept over sulphuric acid probably cause the spiracles to open, and therefore increase the loss of water. As an experimental animal I required an insect which is capable of prolonged fasting, so that it should have a low rate of metabolism; in such an insect loss of weight may be regarded, as a first approximation, as nearly equal to loss of water. An insect which is dormant owing to hibernation or æstivation is not suitable, because it cannot be exposed to a variety of temperatures and remain dormant. A pupa, which is apparently dormant, is also unsuitable, because at this stage metabolism is rapid and also complex, owing to the conflicting processes of histolysis and histogenesis (see Krogh, 1914).

The insect which seems to satisfy these requirements is the meal-worm, the larva of the beetle *Tenebrio molitor*. It is easy to obtain at all times of the year; it is able to survive prolonged starvation, and after it has starved for 2 or 3 days it lies quiet even at so high a temperature as 30° C., so that its metabolism is not increased by movement. It is, moreover, able to live at a wide range of temperatures and humidities.

* The same graph (fig. 1) may be used in connection with the dew point, which I mention because it is so frequently employed in physical tables, though it is not in itself of interest to biologists. Take for instance point E; a reference to the vertical scale shows that it has a vapour pressure of 23 mm. of mercury. The point F has the same vapour pressure, and lies on the saturation curve AB corresponding to a temperature of 24·5° C., which is the dew point of E.

† Any particular mixture of sulphuric acid and water in a closed space has over it an atmosphere of a particular *relative* humidity, and this remains almost constant over the range of temperature here considered.

The principal objection to the meal-worm as an experimental animal is that it is specialised, having a remarkable ability to economise water and resist drying. It is this ability, one may suppose, which has enabled the Tenebrionid beetles to be so successful in colonising the deserts of all parts of the globe (Buxton, 1923, 1924).

At first I assumed that much information would exist with regard to the physiology of the meal-worm. But this was not so, and my investigation proved extremely tedious because so little is recorded.

The meal-worms were reared according to Arendsen Hein's method. His paper is important, and should be consulted by anyone who has occasion to use these insects. All stages of the insect were kept in glass jam pots, at a temperature of 23° C. The larvæ should not be kept in pure bran, in which they develop slowly and with a high mortality. It appears, though the matter has never been investigated in detail, that they require the addition of some form of animal fat, and this may be conveniently given by adding one-fifth of ground-up dog biscuit to the bran. It is convenient to give the larvæ slices of turnip, which they appear to use as a water supply, but this must be done sparingly because the growth of the mite *Tyroglyphus farinæ* is encouraged if the bran becomes damp. Larvæ which were to be used experimentally were kept at 23° C. without turnip, as it was found that the water content of individuals became more regular if turnip was not given. From time to time larvæ can be removed to clean bran by a sieve, the bottom of which should consist of perforated metal and not of woven wire. Pupæ should be removed at least once a week and put into jam pots by themselves, otherwise they are gnawed by the larvæ. The adults feed on the mixture of bran and dog biscuit, and they should also be given turnip, for without it they frequently bite one another and their eggs. They should be given pieces of green baize or some roughly woven material, and they will deposit many of their eggs in the interstices of it, but others are dropped loose in the bran ; as they are sticky when they are first laid, it is extremely difficult to discover them.

For experimental purposes larvæ weighing 80 to 110 mg. were selected. It would be convenient to use the largest larvæ, but pupation occurs if larvæ beyond 110 mg. are starved ; indeed it occasionally occurs in larvæ weighing as little as 100 mg. This is remarkable, for a meal-worm provided with food will grow to 220 or even 250 mg. before pupation. The larvæ were kept in separate wide tubes in the desiccators, the individuals being numbered and weighed at intervals. In the subsequent construction of means, curves, etc.,

only those individuals were used which survived to the end of an experiment, and which had not pupated.

In much of the early work it was found that if an experiment were repeated an inconsistent result was generally obtained. Some of this inconsistency may be avoided by a careful attention to methods of rearing, and all the facts used in this paper were obtained from a single batch of larvæ. But in spite of every care, irregular results still occur (see for instance fig. 6), and it appears that this is due to the great differences that exist in the water content of individual larvæ. The spot diagram, fig. 2, shows the proportion of dry matter

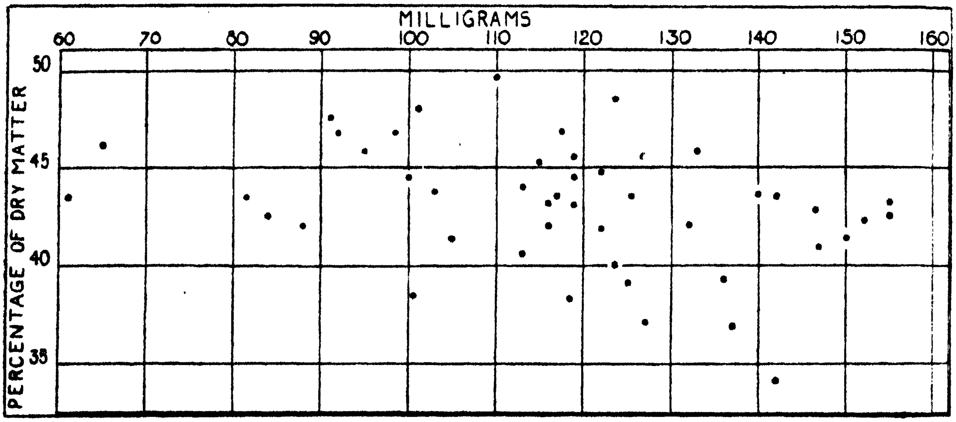

Fig. 2.—Showing the relation between the original weight of 46 meal-worms, all from the same culture, and the percentage of dry matter in them.

in 46 larvæ which had not been starved; they were all taken from the same culture, weighed, and dried at 100° C. at a reduced pressure over phosphorus pentoxide. These larvæ came from the same batch as those used in the experiments described below. It is clear from the diagram that between 80 and 160 mg. the proportion of dry matter bears little or no relation to the weight, and that the scatter about the mean is very great. Taking only the 38 individuals between 80 and 140 mg., the mean proportion of dry matter in them is 42·868 per cent. (P.E. ± 0·491 per cent.).

It seems then that we cannot produce a standard meal-worm. Owing to this difficulty the relations between the insect and the humidity of the atmosphere cannot be analysed with a very high degree of precision, and I think that further advance in knowledge must come from some other method, or the use of a different insect.

If larvæ which weigh from 80 to 110 mg. are starved, there is no relation

between the weight of the larva and the rate of loss of weight. The loss of weight at moulting is less than half a milligram; we must assume that ecdysial fluid exists, but the quantity of it is remarkably little. If a meal-worm moults in the course of an experiment, the larva and its skin may be weighed on subsequent occasions, and no correction need be made. But it is better to weigh and remove the skin, as the larvæ eat their cast skins if they are kept at 90 per cent. relative humidity, though they never do so at lower humidities. On several occasions fasting larvæ have moulted twice during an experiment—a curious fact, the bearing of which upon our understanding of ecdysis is obscure.

All weighings were accurate to 0·5 mg., about 0·5 per cent. of the weight of the larva. Fæces were thrown away, the loss or weight being inconsiderable, and the growth of moulds in damp atmospheres being avoided.

Metabolism of Fasting Larvæ.

Preliminary work showed that the meal-worm will live for many weeks at room temperature without food, in a damp or a dry atmosphere. For instance, a batch of 10 was kept over strong sulphuric acid, and the first death occurred on the 210th day from the beginning of the experiment. The weight of the batch was then 60 per cent. of what it had been at the start. Two parallel experiments over water were performed at the same time. In one of them the weight of the batch fell to 92 per cent. by the 17th day, and then remained very nearly constant until the 150th day, when the first death occurred. In the other batch, weight fell in a similar way and then became constant at just over 90 per cent., the first death occurring on the 207th day. The constancy of weight in damp air, during prolonged starvation, is apparently due to the larva's ability to hold some part of the water produced in metabolism, as is shown below. From these three observations it follows that metabolism in the fasting meal-worm, at any rate at room temperature, must be very low.

One would, in fact, be almost justified in assuming that loss of weight was a measure of loss of water; but I have investigated the point more fully. Air from a cylinder was freed from carbon dioxide and dried, and then passed slowly into an incubator and through U-tubes of calcium chloride, partly to assure its being dry, partly to give it an opportunity of taking up the temperature of the incubator; it was actually shown that the air at this point was at the same temperature as the incubator. The air passed on through a flat-bottomed container, which held 20 or 30 meal-worms weighing roughly 100 mg. each; the container had a cross-section of 3 sq. inches, and the flow of air

was only a few litres per hour, so that loss of water from the insects was not increased by the rate of flow of the air. The air then passed through two Landsiedle U-tubes of calcium chloride, which absorbed the water produced by the meal-worms. From this point the air bubbled through two absorption tubes filled with potash, which collected the carbon dioxide produced by the insects.* From the potash the air passed through two U-tubes of calcium chloride, where the water evaporated from the potash is absorbed. Finally it bubbled through strong glycerine, which acted as a trap and a tell-tale. All the calcium chloride used had been previously saturated with carbon dioxide; all rubber connections were of thick-walled tubing and were as short as possible; the U-tubes and absorption tubes had well-lubricated ground joints. In a blank experiment, air was bubbled for 100 hours through the apparatus, which contained no insects; no weight was gained or lost in the apparatus as a whole. The whole apparatus fitted into a frame of "meccano," designed to slide into the incubator.

It will be seen that this apparatus enables one to weigh the carbon dioxide daily, and to avoid the necessity of making volumetric measurements of the gas. The increase in weight in the successive parts of the apparatus gives one the weight of water given off by the insects, and the weight of carbon dioxide. After the first few days it was found sufficient to weigh the parts of the apparatus twice a week. Table I shows the daily production of carbon dioxide expressed as milligrams of carbon per gram of insects, at 23° C. and 30° C. The calculations are based on the weight of the larvæ on the first day of the experiment. It is clear that the loss of weight which may be attributed to loss of carbon is very little.† In fact I have felt justified in neglecting it in the curves which follow except in figs. 6 and 7. It will be shown later that metabolism in dry

* The first absorption tube should contain very weak potash—about 5 per cent.—so that the carbonates produced are held in solution and do not accumulate at the point where the air enters the potash. The second absorption tube should contain potash saturated at room temperature, partly in order to ensure that the last trace of carbon dioxide is absorbed, partly to hold the water which has evaporated from the first tube. In this way we avoid passing much water to the calcium chloride, which may therefore be used for a number of days without requiring renewal or weighing.

† In considering the loss of weight, we may take it as certain that the carbon has come from the insect, but the oxygen in the carbon dioxide, or at any rate a part of it, has come from the air which the insect has respired. For the same reason, the water collected from the insects includes water of metabolism, and also water evaporated from the surface of the insect and from the inside of the tracheal system. In the absence of detailed studies on the respiratory quotient, after various periods of fasting, we cannot distinguish between water formed in metabolism and that evaporated.

air, which is what this experiment measures, is more rapid than it is at the same temperature in moist air. The losses of carbon given in Table I may therefore be regarded as maxima.

Johansson has experimented with fasting meal-worms, placed in a Thunberg microrespirometer at 20° C. He finds that larvæ which had been unfed for 4 to 24 days give off between 100 and 200 cu. mm. of carbon dioxide, per gram,

Table I.—Showing weight of carbon expired, as milligrams, per 24 hours, per gram of meal-worms; carbon is calculated from weight of carbon dioxide collected; results are reckoned on weight of larvæ on first day of experiment; fasting larvæ from 80 to 110 mg. are used.

Day.	Milligrams of carbon.	
	23°.	30°.
1	5·2	9·0
2	4·1	8·5
3	3·3	7·9
4	3·1	7·4
5	3·0	6·8
6	2·9	6·3
7–11	2·73	4·8
12–16	2·32	?
17–30	2·18	?

per half-hour. These figures correspond to 2·6 and 5·2 mg. of carbon per gram per 24 hours. His figures, which are not very consistent with one another, are higher than mine; this is probably because his larvæ, in a short experiment, are awake and active, whereas mine lay quite motionless except when weighing was in progress.

Studies in Loss of Weight.

We can now consider the loss of weight of the meal-worms kept at a known temperature and humidity. Fig. 3 shows the type of curve which is obtained; it gives the mean loss of weight of 5 larvæ at a temperature of 30° to 31° C., and a relative humidity of 0 per cent. It shows also the loss of weight of the individuals, distinguished by different symbols. This curve is a fair sample of those obtained at other temperatures and at humidities ranging from 0 to 80 per cent. At higher humidities anomalous results were obtained, which are discussed below (fig. 6). It will be observed that throughout the experiment the extreme values for individuals are 2½ per cent. above and below the mean;

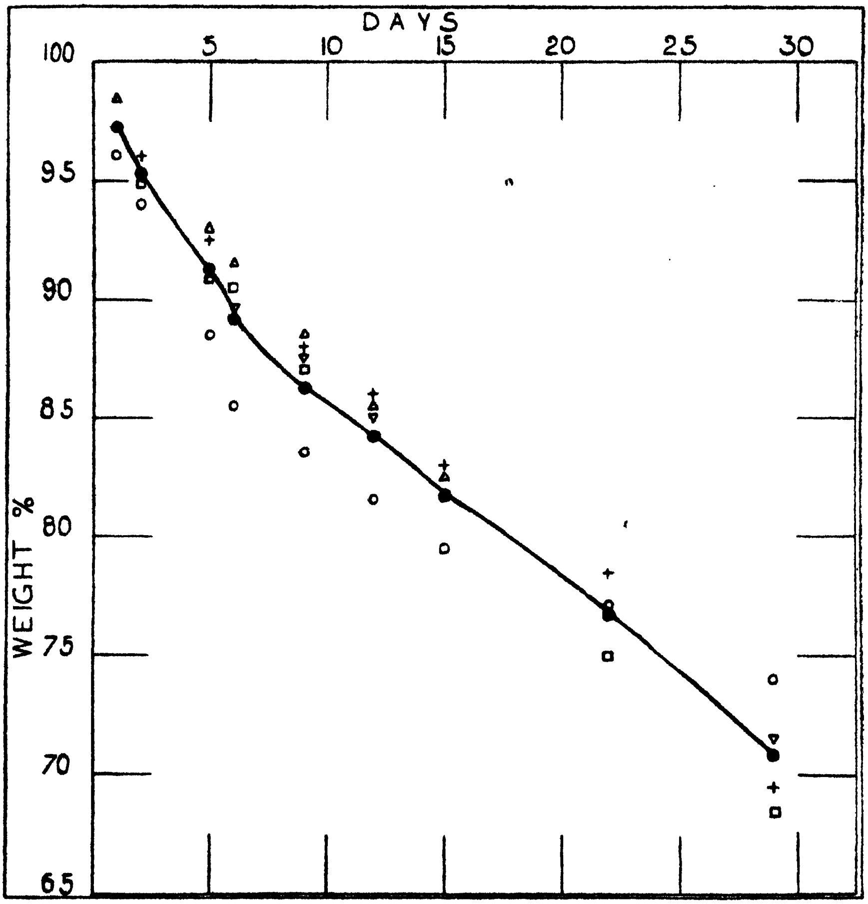

FIG. 3.—Showing loss of weight per cent. of five fasting meal-worms kept at a relative humidity of 0 per cent., and 30° to 31° C., for one month. The curve connects the mean values; the individual larvæ are distinguished by symbols.

indeed the great majority of them are within 2 per cent. At lower temperatures and in less dry air the loss of weight and the scatter are less.

In figs. 4 and 5 I give similar curves which show the mean loss per cent. for batches of larvæ kept at various degrees of relative humidity, and at 23° or 30° to 31° C. The experiments all lasted 4 weeks. If fig. 4 is considered, it will be seen that all the curves show a rapid loss of weight at first, and that in most of them the rate of loss again increases after about the 20th day; but the shapes of the curves are different from one another. Inasmuch as insects kept in dry air lose weight much more rapidly than those in moist air, it is clear that loss of weight is largely determined by loss of water; but the loss

is not directly proportional to the humidity of the atmosphere, no matter how that humidity is measured; this follows from the fact that the curves for 0, 30 and 60 per cent. are not equidistant. The curves obtained at the same relative humidities and a temperature of 30° C. (fig. 5) should be compared with those at 23° C. The same general conclusion will be drawn: loss of weight is determined by the dryness of the atmosphere, but is not proportional to it.

Special attention may be directed to the results obtained in very dry and very moist air. It will be observed that in dry air the loss of weight is almost identical at 23° and 30° C., indeed the loss is a little less at the high temperature. Neglecting the slight difference, we may perhaps explain the similarity of the curves by supposing that the loss of water cannot proceed more rapidly than a certain rate. The same explanation would cover the fact that at 30° C. the loss for the first 10 days is the same at 0, 30 and 60 per cent. humidities.

We may now examine the weight of larvæ maintained in very damp air. Experiments were carried out both at 23° and at 30° C., at relative humidities of 70, 75, 80 and 85 per cent. The curves were very similar, and only those for 80 per cent. are shown in figs. 4 and 5; it will be seen that the loss was less than at 60 per cent., as would indeed be expected. But in experiments conducted at 90 per cent. relative humidity, an unexpected result was obtained. Fig. 6 shows that the mean weight of seven larvæ, maintained at 30° C. and a relative humidity of 90 per cent., fell slightly for the first 2 days, and then rose, remaining between 100 and 105 per cent. from the 9th day till the end of the experiment. The weights of the individual larvæ alter in the most remarkable manner; two were consistently below 100 per cent.; six were consistently above 100 per cent. (after the first 2 days), and ranged as high as 118 per cent. in one instance; a single larva fell to 94 per cent. on the sixth day, had risen to 107 per cent. 3 days later, and finally reached 114 per cent. on the 23rd day.

The general tendency for weight to rise after the first few days was observed in several experiments at a relative humidity of 90 per cent. both at 23° and at 30° C. The same result was obtained in an experiment in which larvæ were kept at 30° C., and a relative humidity of 0 per cent. till the 7th day; the weight of the batch was then 90·6 per cent. of what it had originally been. The larvæ were then transferred to a relative humidity of 90 per cent., at the same temperature, and the weight rose to 92·1 per cent. on the 11th day, and 92·5 per cent. on the 14th day.

The most probable explanation of all these observations at 90 per cent. saturation is that the starving insect produces more metabolic water than it can liberate into a moist atmosphere ; the only alternative explanation is that the larva is hygroscopic. I shall show later (Table II) that the proportion of water in larvæ which have been starved at a 90 per cent. relative humidity

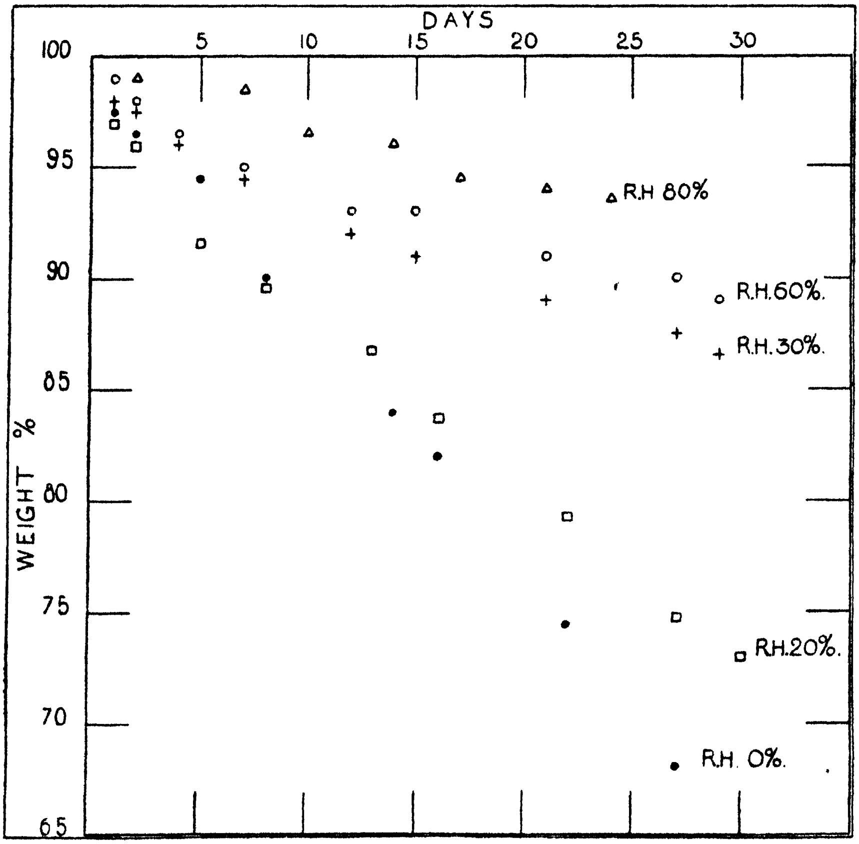

FIG. 4.—Mean loss of weight of groups of meal-worms kept at 23° C., and various relative humidities from 0 to 80 per cent.

is much greater than it is in those starved at lower humidities. As to the inconsistencies (fig. 6) between individual larvæ, I cannot explain them, but they were observed in several different experiments. At one time I supposed that they might be related to the time which had elapsed since the individual's last moult; it seemed possible that water might be more readily lost through chitin soon after moulting than later ; but this idea was shown to be erroneous,

for several larvæ moulted during an experiment without greatly gaining or losing weight.

We conclude, therefore, that losses of weight in atmospheres which are either very dry (0 per cent.) or very moist (90 per cent.) must be considered as special cases.

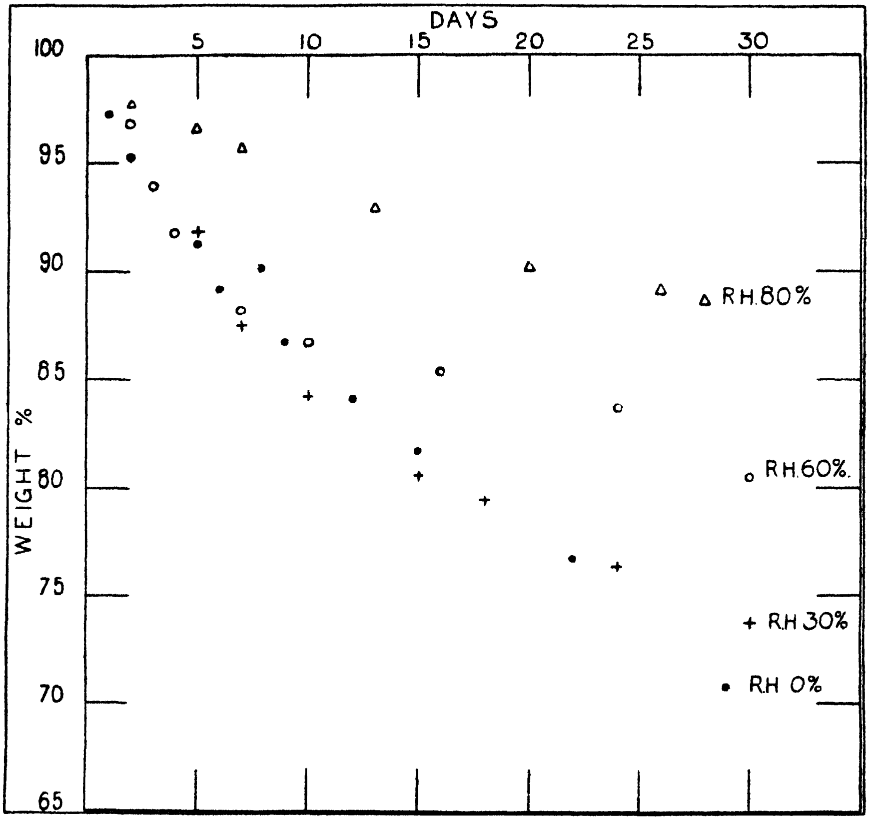

Fig. 5.—Mean loss of weight of groups of meal-worms kept at 30° C., and various relative humidities from 0 to 80 per cent.

So far I have considered the loss of weight at various humidities and a single temperature, either 23° or 30° C. The possibility remains that we could find an appropriate measure of humidity, and compare conditions at different temperatures: we might in this way discover a law for water loss. An inspection of figs. 4 and 5 shows that the same *relative humidity* at different temperatures produces very different results. Except in dry air, loss of weight is

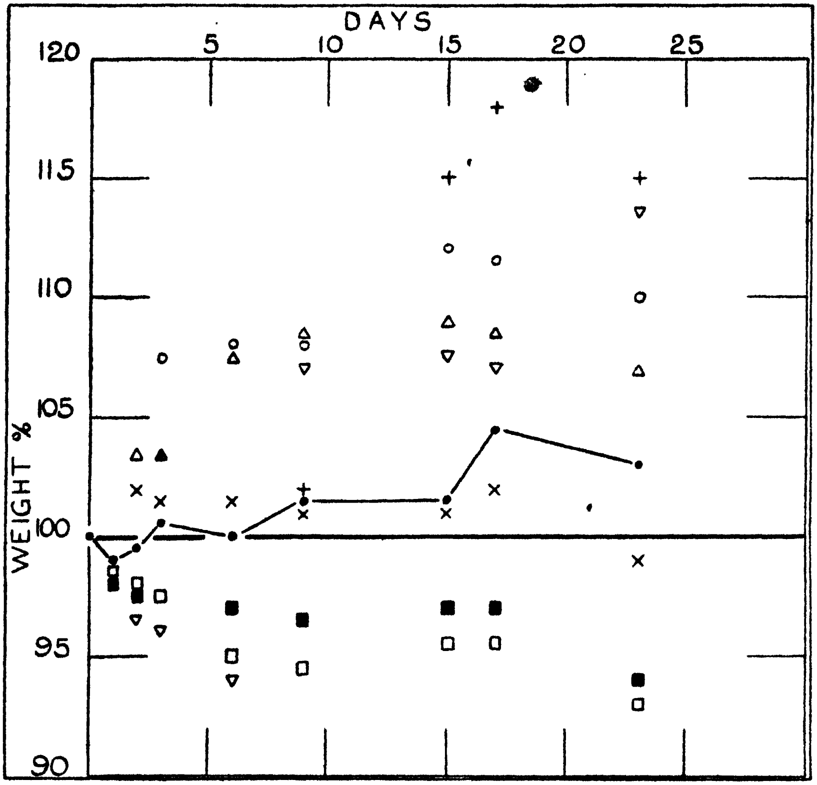

FIG. 6.—Mean weight per cent. (continuous line) of seven meal-worms maintained at 30° C. and a relative humidity of 90 per cent.; percentage weights of the individuals shown by symbols which are different for each larva.

always greater at the higher temperature, as is evident if the curves for 30 per cent., 60 per cent. or 80 per cent. at the two temperatures are compared. It is possible to compare two batches kept at the same *vapour pressure*, for a relative humidity of 90 per cent. at 23° C. has a vapour pressure of 19·5 mm.; this is identical with the vapour pressure of a relative humidity of 60 per cent. at 30° C. (fig. 1). A comparison of the two curves in figs. 4 and 5 shows their essential differences, and I have other unpublished experiments which justify me in saying that if meal-worms are kept at the same vapour pressure and different temperatures, the loss of weight is more rapid at the higher temperature.

Saturation deficiency remains the only probable measure applicable to our case, if indeed a physical measure can be found. In fig. 7 we compare the loss of weight of two batches of larvæ kept at different temperatures and relative

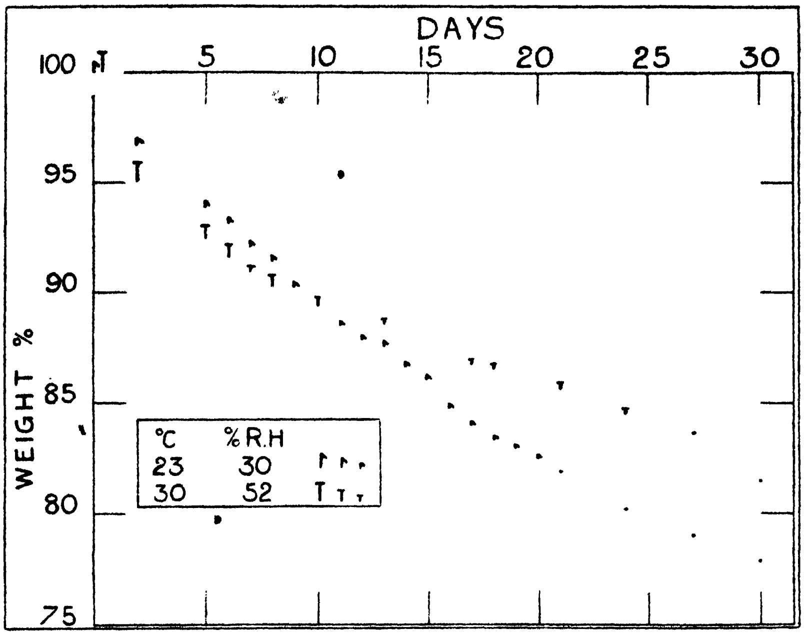

FIG. 7.—Mean loss of weight of two batches of larvæ, both kept at a saturation deficiency of 15 mm. of mercury at 23° and 30° C. The vertical lines show the magnitude of the correction for loss of carbon.

humidities, both at a saturation deficiency of 15 mm. of mercury. Fig. 8 gives a similar example, the saturation deficiency being 21 mm. The curves in both cases have been corrected for loss of weight of carbon, and the magnitude of the correction is shown by the vertical lines on the graph. Though the curves do not precisely coincide, it will be noticed that the correction tends to bring them closer together during the first 10 or 20 days; during this period the loss of weight and also the correction for metabolism is greater at 30° C. than at the lower temperature. In the latter part of the month loss of carbon is so little that I make no correction for it, and the curves cross and diverge in an unexpected manner, for the loss of weight at the higher temperature becomes less than at the lower.

It would, therefore, be true to say that if fasting meal-worms are kept a the same saturation deficiency, and different temperatures, their loss of water is nearly identical. But this is only correct for about the first 10 to 20 days of the experiment; and it is incorrect if the saturation deficiency is very little (fig. 6) or very great. But even if we exclude the very dry and very moist conditions we cannot use saturation deficiency as a measure of loss of water

from larvæ kept at different humidities and the same temperature; in other words, the larvæ do not conform to Dalton's law of evaporation, which states that, under otherwise identical conditions, loss by evaporation is directly

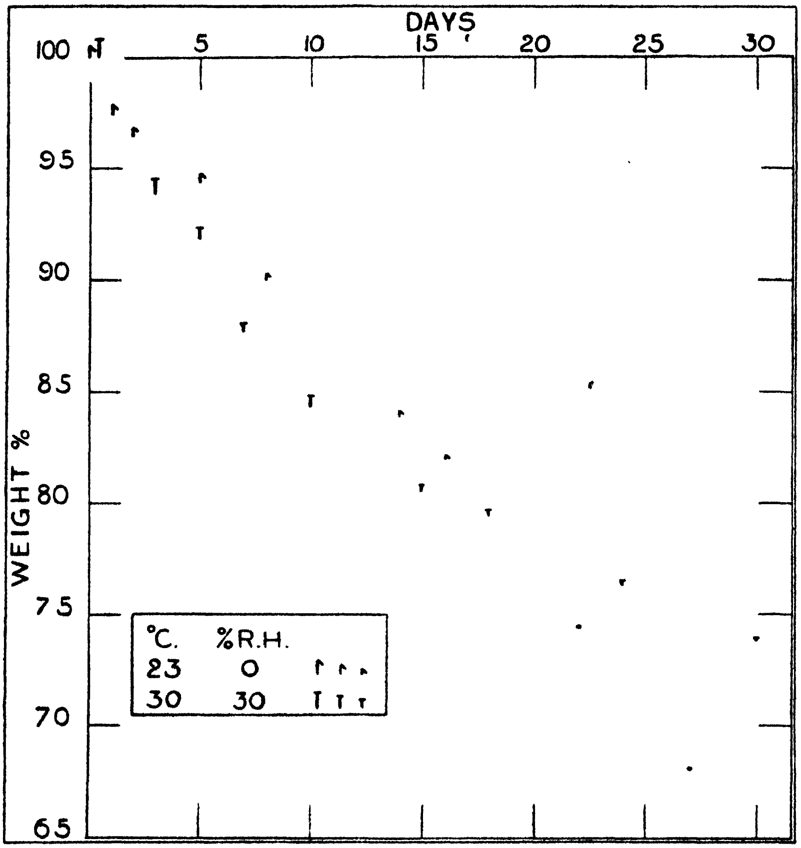

FIG. 8.—Mean loss of weight of two batches of larvæ, both kept at a saturation deficiency of 21 mm. of mercury at 23° and 30° C. The vertical lines show the magnitude of the correction for loss of carbon.

proportional to saturation deficiency. That this does not hold good for meal-worms may be shown by calculating the ratio $\frac{\text{Loss of weight}}{\text{Saturation Deficiency}}$ from meal-worms kept at 23° C. (fig. 4). The following results are obtained :—

Relative humidity.	Day 10.	Day 20.	Day 30.
60 per cent.	0·71	1·00	1·35
30 per cent.	0·48	0·72	0·97

Had Dalton's law been applicable the figure for the two humidities on any one day would have been identical. But the ratios show that the loss at 30 per cent. is less than it would have been had the law been true. The same is true if the ratios are calculated from facts collected at 30° C. (fig. 5).

The fact that loss is less at lower humidities may be due to one or both of two factors. It is probable that the larva reduces loss of water from within the tracheal system by closing its spiracles when it is put in a dry atmosphere. Moreover, when the external atmosphere is moist, there is little difference in humidity between the air in the tracheal system and that outside the insect; but when the external atmosphere is dry, the air in the tracheæ is much moister than that on the insect's surface, so that in this case the loss of water from the tracheal system becomes relatively less important. The analogy between the insect and the green leaf, loss of water from which is regulated by closing stomata, will be apparent.

We have discussed loss of weight and shown that it is nearly identical with loss of water. We must now investigate the proportion of water and of dry matter which remains in the larva after it has starved at a certain humidity for 4 weeks. The larvæ came from a stock which contained $42 \cdot 868 \pm 0 \cdot 491$ per cent. of dry matter. Those which were maintained at a relative humidity of 90 per cent. (Table II) rose 3 per cent. in weight, and the proportion of dry matter at the end of 23 days was $34 \cdot 78 \pm 0 \cdot 78$ per cent.; that is to say, they had produced more water than they had been able to dissipate into the nearly saturated air. The larvæ kept for 4 weeks at lower humidities, from 60 per cent. to 0 per cent., had maintained their water content very nearly constant for the 4 weeks of the experiment. Indeed the difference between the proportion of dry matter in the larvæ which had lived at 60 and at 30 per cent. humidity is not statistically significant, as can be seen from the Probable Errors; those kept at 20 per cent. and 0 per cent. humidity contained a rather higher proportion of dry matter, but the difference between these two batches is not significant. On the whole, one may say that the proportion of solids is nearly the same in larvæ starved at any humidity from 60 to 0 per cent., and that it remains very near to what it must have been at the beginning of the experiment.

This almost precise maintenance of the proportion of dry and wet matter after a month's starvation at relative humidities between 60 and 0 per cent. is remarkable in view of the great differences in loss of weight. The mechanism by which it is brought about is suggested by the figures in the last column of Table II. In that column the dry weight of the larvæ at the end of the experi-

Table II.—Larvæ are kept 4 weeks at 23° C., and various relative humidities, and gain or lose weight. The table gives also the percentage of dry matter in the larvæ at the end of the experiment, and the same expressed as a percentage of the original live weight. All larvæ were weighed individually.*

Number of larvæ.	Relative humidity.	Mean weight as percentage of original weight.	Dry matter.	
			As percentage of final weight.	As percentage of original weight.
	per cent.			
5†	90	103	$34 \cdot 78 \pm 0 \cdot 78$	$36 \cdot 44 \pm 0 \cdot 66$
5	60	89	$42 \cdot 16 \pm 0 \cdot 40$	$36 \cdot 84 \pm 0 \cdot 42$
4	30	86·5	$42 \cdot 02 \pm 0 \cdot 51$	$36 \cdot 125 \pm 0 \cdot 76$
7	20	73	$44 \cdot 24 \pm 0 \cdot 54$	$31 \cdot 30 \pm 0 \cdot 63$
5	0	68	$44 \cdot 0 \pm 0 \cdot 30$	$30 \cdot 74 \pm 0 \cdot 49$

† Killed and weighed on 23rd day.

ment is shown as a percentage of what their live weight had been at the beginning. The differences between larvæ starved at 90, 60 and 30 per cent. humidity are not significant. But the larvæ kept at 20 per cent. and at 0 per cent. had lost much more dry matter during the experiment than had those maintained in the three higher humidities. It seems, therefore, that if a meal-worm is starved in dry air, it maintains a due proportion of water in its body by making inroads on some reserves, and so producing and using water of metabolism.

I have, I think, made it clear that the loss of water from a meal-worm into the atmosphere is very complex, even if the insect is not fed during the experiment.

It is a pleasure to acknowledge the kindness of Mr. David Lewis, who helped with weighing, Mr. H. S. Leeson, who drew the graphs, and Dr. J. S. Haldane, who read and criticised the manuscript.

Summary.

The paper discusses the results obtained by keeping meal-worms at 23° and 30° C. and in atmospheres of various humidities. Even in dry air and at 30° C. fasting larvæ generally live a month. Metabolism must therefore be low,

* The larvæ whose dry weight is here considered are identical with those whose loss of weight is shown graphically in fig. 4.

and this is shown to be the case by weighing the daily production of CO_2 from fasting larvæ kept in dry air. Loss of weight of larvæ is therefore very nearly the same as loss of water. It is not possible to produce a standard meal-worm with a constant proportion of water in it.

The loss of water from a fasting meal-worm is complex. For humidities below 90 per cent., the loss is nearly identical in larvæ kept at 23° and 30° C. at the same saturation deficiency. But neither saturation deficiency nor any other measure of atmospheric humidity explains the loss at several humidities and any one temperature. It seems that there is a definite limit to the amount of water which a larva can lose in a day ; also that in nearly saturated air the larva produces more water of metabolism than it can get rid of.

It is shown that the larva can maintain the proportion of water in its body nearly constant, during a month's fast, at humidities from 0 to 60 per cent. It appears that it is able to do this by consuming some stored substance and holding the water produced in metabolism.

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The Corpora Lutea of the Mouse, with Special Reference to Fat Accumulation during the Œstrous Cycle.

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[Plates 37–39.]

Introduction.

Although the histological literature on the corpus luteum is very extensive, a description of cellular and fat changes in the organ, which distinguishes between the corpora lutea of ovulation, pseudo-pregnancy, pregnancy, and lactation where these are differentiated, is still lacking for some laboratory animals. In view of experimental work now in progress, it was thought that a short account of the mouse corpus luteum on these lines might be of value.

In the absence of pregnancy, old corpora lutea persist for a considerable period in the mouse ovary, and after the initial signs of cellular degeneration, which are not always very obvious, further changes are slow to occur. With suitable histological methods, however, it is possible to distinguish, even after they are fully developed, the corpora lutea belonging to the last one and sometimes two or more œstrous periods.

Previous papers containing descriptions of fat changes in the corpus luteum include Horrenberger (9) and Watrin (23, 24) on the human corpus luteum; Goormaghtigh (8) on the corpus luteum of pregnancy in the dog; Long and Evans's monograph (10) on the rat; Corner (4–6) on the sow; Mulon (12) on the guinea-pig; Van der Stricht (22) on the bat; and Drips (7) on the spermophile.

The histochemistry of the fats of the corpus luteum is discussed in detail in the paper by Goormaghtigh (8). This author concludes for a number of reasons that the deposit of histologically stainable fats in the lutein cell indicates a slowing down of the metabolism of the cell, but not necessarily the beginning of its degeneration. Further, Goormaghtigh discusses the nature and significance of the cytoplasmic substances which, after osmic acid fixation, have an affinity for iron hæmatoxylin; he concludes that these "siderophil" masses represent complex fatty substances associated with proteins. Having investigated similar substances in the adrenal cortex, he believes that they represent

an intermediate phase of the secretory product, which if fixed, in osmic acid, blackens in the inactive or reserve condition, but remains colourless in an actively secreting cell. This affinity of the cytoplasm for iron hæmatoxylin persists in the mouse corpus luteum after the sections have been treated with turpentine to remove the osmicated fat prior to staining. The siderophil substance is commonest in cells which lack osmicated fat, but it is also found in association with it, both in lutein cells and in the vascular tissue ramifying among them.

In the present paper, the term "lutein cells" will be restricted to the cells originally forming the follicular epithelium, while, to avoid ambiguity, "theca interna cells" will apply only to those cells containing coarse fat which border the ripe follicle and the young corpus luteum. Unless otherwise stated, the terms "fats" and "lipoids" will refer to the substances seen in sections through the corpus luteum, prepared according to one or other of the methods given below.

After a certain period of growth and functional activity (the duration of which appears to be approximately constant for each type of corpus luteum, except that of lactation), as the next ovulation approaches, fat begins to accumulate in the lutein cells. About this stage it is common to find in sections a much greater quantity of fats and lipoids on the outside, and an abrupt transition to an inner less fatty region. The term transitional is here applied to these corpora lutea. The condition is most marked in sections prepared by Ciaccio's method (Plate 37, fig. 4). Long and Evans (10) have described a similar condition in the corpora lutea of rats. In old corpora lutea, from which fat and lipoids are disappearing after the next ovulation, the transitional appearance is also sometimes seen, but it is less marked. This difference between the outer and inner regions of the corpus luteum in the rate of accumulation and disappearance of fat can probably be attributed to the vascularization being less efficient in the outer parts—possibly a result of developmental conditions.

The fat and lipoids in the stroma of the ovary (excluding those which can be distinguished as fragments of old corpora lutea), vary considerably in different mice. No connection could be traced between their appearance at any given stage of the cycle and that of the corpora lutea, in whose formation they do not appear to take part, as has been described in the bat (22). The only general conclusion which it seems possible to draw from the material is that fat and lipoids are most abundant in the ovarian stroma of young mice (contrast figs. 1 and 9).

Material and Technique.

The material for this work consists of the ovaries of 40 normal albino mice ; of these, 11 were unmated, 10 pseudo-pregnant, 17 pregnant, and 12 post-parturition. In 4 cases, or 10 per cent. of those studied, the last œstrus had been unaccompanied by ovulation. The different stages of the cycle were dated by observation of the vaginal smear, parturition, or the finding of the vaginal plug, due to copulation.

The œstrous cycle in the mouse has been described by Allen (1) and Parkes (15). In the unmated animal ovulation occurs every 5 or 6 days, at the beginning of the œstrous period (2), which lasts about 1 day. Examination of the vaginal smear enables the œstrous cycle to be divided into four stages—diœstrus, pro-œstrus, œstrus and metœstrus—the first lasting 2 days and the others about 1 day each. After sterile copulation the mouse becomes pseudo-pregnant and œstrus does not recur for 10 or 12 days. During pregnancy both ovulation and œstrus are suspended. There is an œstrous period within 24 hours of parturition, but not subsequently for 3 or 4 weeks, if at least three young are suckling. With less than three young the return to the normal cycle after parturition is more rapid (15).

It is generally agreed that the corpora lutea have ceased to be functional once the next œstrus has occurred ; in the rat and mouse (19, 20) the corpora lutea of ovulation apparently never become functional, the diœstrous period being so short. There is also histological evidence of corpus luteum degeneration some time before the next œstrus occurs, a condition to which reference is made later, in the discussion.

Histological Technique.

The ovaries were dissected free of fat, and as a routine method, one ovary from each mouse was fixed in Flemming's chrome-osmium fluid with acetic acid for about 24 hours, while the other was placed in Ciaccio's bichromate-formol-acetic acid for 48 hours and then transferred to 3 per cent. potassium bichromate for 24 hours. After both methods of fixation the ovaries were washed overnight in running water, dehydrated and embedded in paraffin. In almost all cases complete serial sections were cut from both ovaries at 7 μ. Flemming-fixed sections were mounted from different parts of the ovary, unstained, in Farrant's glycerine medium, to show the osmicated fats. After Ciaccio fixation, sections were stained in Scharlach R or Sudan III and similarly mounted. Ciaccio's method is believed to stain mixtures of cholesterol and neutral fats, and also certain phosphatids and lecithins, while osmic acid is said to blacken olein and oleic acid, and mixtures containing a sufficient amount of these substances, the black, brown, or yellow colour produced depending upon their quantity (14). When ovaries from the same mouse were compared the two methods gave generally corresponding results. Slides from both ovaries were stained in iron hæmat-

oxylin, those from Flemming-fixed material being previously placed in turpentine to remove the osmicated fat. A few ovaries were fixed in Bouin and variously stained for studying the reticulum.

Corpora Lutea Measurements.

In order to obtain an expression for the size of the corpora lutea of a single cycle, two diameters at right angles to each other were measured across each corpus luteum at its maximum area in section; the average of these results is given as the mean diameter of the group. As regards old corpora lutea, a certain amount of flattening seems to take place and the mean diameter is larger in proportion to the true size of the corpus luteum than in the most recent ones. It decreases at the end of the functional period, but less rapidly than the size of the cells.

Cell size in different corpora lutea was compared by means of a Reichert microscope fitting, which enables slides under two microscopes to be focussed into the same field. Direct comparison of corpora lutea in two ovaries is thus possible. This is more satisfactory than measurements of a limited number of individual cells, owing to irregularities and variations in size. Cell measurements were undertaken, however, in order to provide an approximate record of the lutein cells at different stages. The data are summarized in Table I (p. 588).

The Corpus Luteum of Ovulation.

The early stages of corpus luteum development will be described elsewhere, with special reference to the fate of the theca interna cells which surround the corpus luteum immediately after ovulation.* The present writer, in general agreement with Sobotta (21), holds that the theca cells in the mouse do not persist as such. Other authors, including Corner (4), state that, in the mouse and rat, theca cells can no longer be distinguished as such in the fully formed corpus luteum. As regards the fibrous elements in the corpus luteum, a study of its development and histology in the mouse confirms Corner's view (5) that the reticulum is produced by the endothelial cells; when the corpus luteum has reached its maximum size there can be distinguished only lutein cells—the original follicular epithelium hypertrophied—and vascular tissue.

About 8 hours after ovulation the corpus luteum has a mean diameter of 420 μ; 12 to 18 hours later, at metœstrus, this has enlarged to 560 μ owing to the increase in the size of the lutein cells. The vascular tissue is still proliferating and its cells, owing to their siderophil cytoplasm, stand out conspicuously among the lutein elements (fig. 5). At the edge of the corpus luteum faint osmicated granulations are beginning to appear in the lutein cells, but elsewhere there is no fat in the cells (fig. 6), though lipoids are present in small amounts throughout the corpus luteum.

From metœstrus till the end of the cycle a rapid increase takes place in the

* Very full discussion of this subject occurs in the corpus luteum literature, including (4), (7), (9), (21) and (22).

fat and lipoid contents of the lutein cells, which undergo further enlargement. At first the fat and lipoid granules are abundant in some cells only, but soon all the lutein elements become uniformly charged with them. The granulation tends to become coarser as the next œstrus approaches (figs. 8 and 9) and the lipoids take a deeper stain. The transitional type of corpus luteum (*cf.* fig. 4) in which the lipoid granulation is heavier in the outer parts is found 2 days after metœstrus and later. In the fully developed corpus luteum the reticular tissue forms a fine fibrous network, radiating from small endothelial nuclei. In a diœstrous corpus luteum (figs. 7, 8), uniformly laden with fine osmicated granules, the branching network, still proliferating, stands out clearly; many of the reticular cells contain fats and lipoids, similar to those in the lutein cells. There seems no reason to believe that these cells have a secretory function; presumably the fats in their cytoplasm are derived from the lutein cells, and represent a phase of the secretory product being assimilated by the vascular system. These fats, in association with proteins (forming the siderophil substance of Goormaghtigh (8)), when present cause the cytoplasm of the cells to appear black in iron hæmatoxylin-stained sections. The lutein cell cytoplasm in the corpus luteum of ovulation stains only lightly, after treatment with turpentine.

At the time of the next œstrus the mean diameter of the corpus luteum is about 600 μ; the lutein cells have a diameter of about 12 μ and their nuclei 7–8 μ; thus the nuclei are large in proportion to the size of the cells. The largest globules of osmicated fat in the lutein cells have a diameter $2–2 \cdot 5\ \mu$, but most of the fat is still in granular form (fig. 9).

By the time the next ovulation has occurred the fat granulation in the old corpora lutea has become coarser and less distinct, as though the minute fat droplets were tending to coalesce. Gradually the total fat diminishes, till, 2 days after metœstrus, the corpora lutea of the last cycle contain hardly any fat or lipoids. The loss of fat is accompanied by a reduction in the average size of the nuclei and cells, and a loss of definition of the cell boundaries; the nuclear and cell diameters become approximately 3–4 μ and 6 μ. Although this shrinkage takes place soon after the end of the cycle, it is not a prelude to the immediate degeneration and disappearance of the old corpora. In the absence of pregnancy the latter persist for several weeks, and only the most recent and the oldest corpora can be distinguished with certainty. These often contain large globules of osmicated fat irregularly distributed (*cf.* fig. 2). Since about five corpora lutea normally develop after each œstrus period, a single ovary often contains twenty or more of these bodies (*cf.* fig. 1).

The Corpus Luteum of Pseudo-pregnancy.

After sterile copulation the interval before the next œstrus is prolonged to 10 or 12 days, as compared with the 5-day cycle in the unmated mouse (15). The lengthening of the diœstrous period is correlated with the greater development of the corpus luteum, which becomes functional (19). It grows larger than the corpus of ovulation and shows histological differences from it. The pseudo-pregnant mice, of which the corpora lutea are described below, are dated from the finding of the vaginal plug in the morning, indicating copulation during the previous night.

At 3½ days pseudo-pregnant the corpus luteum differs conspicuously from that of ovulation, in that the lutein cells are fat-free; their nuclei are also rather smaller (Table I). At this stage the mean diameter of the corpus luteum of pseudo-pregnancy is 612 μ. At 4 and 5 days pseudo-pregnant there is little change in the size of the nuclei and cells, but faint fatty granulations have begun to appear in the cytoplasm. These serve to distinguish the corpus luteum of pseudo-pregnancy from that of pregnancy of the same age; in the ovaries examined the latter has a smaller mean diameter.

The corpus luteum of pseudo-pregnancy reaches its maximum size about 6 or 7 days after copulation; the mean diameter is about 720 μ. Since the mean diameter of the corpus of ovulation when fully developed is commonly about 600 μ, it has increased by 10 to 20 per cent., owing to the enlargement of the lutein cells. The nuclei, however, remain smaller than in the corpus of ovulation (fig. 10). At 6 and 7 days pseudo-pregnant a considerable quantity of fatty granulations have accumulated, which at first stain brown rather than black with osmic acid (fig. 11). Corpora lutea from an 11-day pseudo-pregnant mouse showed little difference from the 7-day stage, but others from a 9-day pseudo-pregnancy contained more osmicated fat than those described above. Thirteen days after sterile copulation, when the succeeding ovulation had just occurred, there is a shrinkage of the corpora of pseudo-pregnancy—mean diameter 656 μ—which then contain abundant osmicated fat (fig. 1).

To summarize, it may be said that the lutein cells in the corpus luteum of pseudo-pregnancy differ from those in the corpus of ovulation in the slower rate at which they accumulate fat and in their slightly larger size and smaller nuclei. The vascular tissue ramifying among them appears more regularly developed than in the corpora lutea of ovulation (figs. 7 and 11) and the endothelial nuclei are smaller.

The Corpus Luteum of Pregnancy.

During pregnancy the growth of the corpus luteum (see Table I) and the accompanying changes can be roughly divided into three periods, of which the first lasts till the eighth day after copulation. Up till then the corpus luteum is hardly larger than that of the unmated cycle (and is smaller than that of pseudo-pregnancy), its mean diameter after the third or fourth day being about 600–650 μ. Histologically, it diverges early from the corpus luteum of ovulation, owing to fats and lipoids being almost absent from its cells; in size and shape the latter are rather more regular than those of the unmated cycle and their nuclei are smaller.

The most fatty corpus luteum of this group is that from a 7-day pregnancy; after Flemming fixation it contains uniformly distributed fine brownish granules of osmicated fat, but it is not so fatty as the corpus luteum of a 6-days' pseudo-pregnant mouse (fig. 11) nor so large. By contrast, the 6- and 8-day corpora lutea of pregnancy which were examined were almost fat-free; it is probable that individual variations of this order in the amount of fat, which also occur later in pregnancy, are without functional significance. In its slow initial rate of development (see the diagram, p. 589, and Table, p. 588) the corpus luteum of pregnancy resembles that of lactation, which also has normally a long functional life.

During the second period of pregnancy the corpus luteum reaches its maximum size and persists without showing signs of histological degeneration; this period of functional activity lasts from the eighth till the sixteenth day, namely up to 3 days before parturition. The corpus luteum enlarges rapidly till about the thirteenth day, after which little or no further increase takes place. As the lutein cells increase in size their fat-contents fluctuate slightly.

Between the tenth and twelfth days of pregnancy all old corpora lutea in the ovary undergo rapid degeneration, becoming reduced to fibrous masses containing large globules of osmicated fat (fig. 2). Although this degeneration coincides with the growth period of the corpora lutea of pregnancy, comparison with other ovaries suggests that its occurrence is not due to purely mechanical causes. It contrasts with the gradual degeneration of corpora lutea in ovaries of non-pregnant mice in being far more abrupt, and in affecting corpora lutea of different cycles simultaneously.

By 9½ and 11 days pregnant the mean diameter of the corpora lutea has reached 810 μ; the lutein cells show an enlargement, but their nuclei, as at

8 days pregnant, are only about 6·5 μ in diameter. In the first of these ovaries the corpora lutea contained a slight amount of osmicated fat; in the second, fats and lipoids seemed to be almost absent—an indication of a functionally active condition (8).

At 13 days pregnant (fig. 2), both the nuclei and cytoplasm of the lutein cells show an increase. The nuclei stain only lightly with hæmatoxylin, and their diameter is about 7·7 μ; that of the cells ranges from 17 to 30 μ, the mean being about 23 μ. The corpus luteum has now reached approximately its maximum dimensions; the mean diameter in the ovaries examined is 976 μ—or nearly 1 mm. After Flemming fixation some of the lutein cells appear vacuolated and others contain black granulations (fig. 2); the lipoids after Ciaccio fixation do not stain deeply. At 16 days pregnant the lutein cells show little change, but contain less osmicated fat, though the lipoids have increased.

During the third period of its history, dating from about the eighteenth day of pregnancy, the corpus luteum accumulates fat and gradually shrinks. One day before parturition there is still no reduction in size (mean corpus luteum diameter at 18 days 982 μ), but there is a distinct increase in the fat and lipoid cell inclusions. At parturition the mean diameter of the corpus luteum has shrunk to 864 μ and the lutein cells appear to have contracted, but their boundaries are too indistinct for individual measurements. In the last 24 hours a considerable accumulation of fat has taken place, which is most heavy in the outer region of the corpus luteum (fig. 12); the granulation of fats and lipoids is coarser than at any time during pregnancy, but it is not of the large irregular type found at the end of the unmated cycle or pseudo-pregnancy (contrast figs. 1, 9, 11, 12).

After parturition the corpora lutea of pregnancy, though degenerate, may persist for a considerable time, as in the rat (10). There is some variation in the rate at which shrinkage and loss of fat occur in them; in three mice killed, 1, 1½ and 5½ days after parturition, the corpora lutea have become much smaller and less fatty; their mean diameters range from 732 to 816 μ. In the second of these the nuclear diameter is only 6 to 6·5 μ, and the cell limits indefinite.

In other mice killed during the first week of lactation, the corpora lutea of pregnancy show much less shrinkage and their fatty cell inclusions increase, indicating a less advanced stage of regression. Thus a mouse killed after 4½ days' lactation had corpora lutea the same size as those in the mouse killed at the time of parturition (fig. 3), and large pregnancy corpora lutea (mean

diameter 798 μ) were found also 7 days after parturition in a mouse which did not ovulate at the *post-partum* œstrus.

During the second and third weeks of lactation the diameters of the corpora lutea of pregnancy range from 678 to 750 μ and osmicated fat and lipoids tend to become sparse and irregularly distributed (fig. 4). Thirty-eight days after parturition, 3 days after the end of the lactation diœstrus, the corpora lutea of pregnancy persisted as intact, fat-free bodies, with a mean diameter of 480 μ.

The Corpora Lutea of Lactation.

The cells in the corpora lutea of lactation, formed at the *post-partum* ovulation, remain very small and almost fat-free till about 8 days old, when the mean diameter is 580–600 μ. The cells correspond in size to those in the fully formed corpus lutea of ovulation, but the nuclei are very small (Table I).

Five mice were killed during later stages ot lactation and their ovaries are described below. A mouse with four young lactating was killed 12 days after parturition ; the mean diameter of the corpora lutea of lactation was 660 μ—rather less than the maximum reached by the corpus of ovulation. The lutein cells of the lactation corpora were shrunken and contained considerable quantities of finely granulated fats and lipoids, giving a brownish, blurred appearance after Flemming fixation (*cf.* the rat (10)). In spite of the histological indications of recent degeneration the maximum size of the follicles showed that the next œstrus was not within measurable distance (2).

Another lactating mouse, which contracted septicæmia, was also killed 12 days after parturition, the young having died 1 day previously ; in this mouse, as in the previous one, the corpora of lactation were shrunken (mean diameter 580 μ) ; they contained abundant black osmicated fat in uniform coarse granules ; in the Ciaccio-fixed ovary some of the corpora lutea showed the transitional condition (fig. 4), indicating that the fatty degeneration was only just occurring. As in the previous mouse, none of the follicles showed signs of pro-œstrous enlargement (2).

A third mouse produced seven young at birth, of which she was suckling three when killed, 17 days after parturition. The corpora lutea of lactation had a mean diameter of 640 μ ; the lutein cells are the largest in the lactation series, and correspond both in cell and nuclear dimensions to the maximum size reached in the pseudo-pregnancy series. In these corpora lutea of lactation fat is less abundant than at 12 days *post partum*. Thus, from the appearance of the lutein cells and their fat-content, it may be inferred that histological

degeneration is less advanced in this mouse than in either of the 12-day mice. In the corpora lutea fixed in Ciaccio the transitional condition is again seen. There are no histological signs of the next œstrus.

A fourth mouse gave suck to four young for 21 days, after which they were removed and the mother mated. The removal of the young terminated the lactation diœstrus, and the mouse was killed 3 days later, following copulation. The ovary contained new corpora lutea, about 8 hours old, and corpora lutea of lactation measuring 680 μ in mean diameter. The latter contrast markedly with those previously described, since they contain very little fat (fig. 13). In stained sections, however, the general appearance suggests old rather than recent corpora lutea, the cells being shrunken, and the cytoplasm eosinophil; it seems probable that the fat accumulated at the end of the functional period is now lost, as normally occurs in the life-history of a corpus luteum. The fat granulation is rather finer and more regular than in most old corpora lutea (*cf.* fig. 12), but does not resemble that in the younger corpora of lactation. The lipoid distribution is again transitional, but of a different type from fig. 4.

The last mouse killed during lactation suckled four young for 5 weeks and then experienced œstrus spontaneously for the first time since the *post-partum* ovulation, and was killed 3 days later. The corpora lutea of lactation measured 710 μ; they were fatty, and the transitional condition in the Ciaccio-fixed ovary (as fig. 4) indicated that they had not long ceased to function. The lutein cells were similar in size to those in the last ovary described. The youngest corpora in this ovary, dating from the last œstrous period, were also of the fatty type, being about 3 days old. The corpora lutea of lactation could be distingushed in stained sections by the smaller size of their nuclei, the absence of cell boundaries, etc.

A general consideration of the corpora lutea of lactation shows that in size they approximate to the corpora lutea of ovulation or pseudo-pregnancy. The largest lutein cells seen during lactation (17 days after parturition) were similar in size and nuclear size to those of a 6-day pseudo-pregnant mouse, *i.e.*, the nuclei were smaller in proportion to the size of the cells than in the corpora lutea of the unmated cycle (Table I). The rate of fat accumulation, however, is slower during lactation than in pseudo-pregnancy, since the corpus luteum normally has a longer functional life.

Discussion.

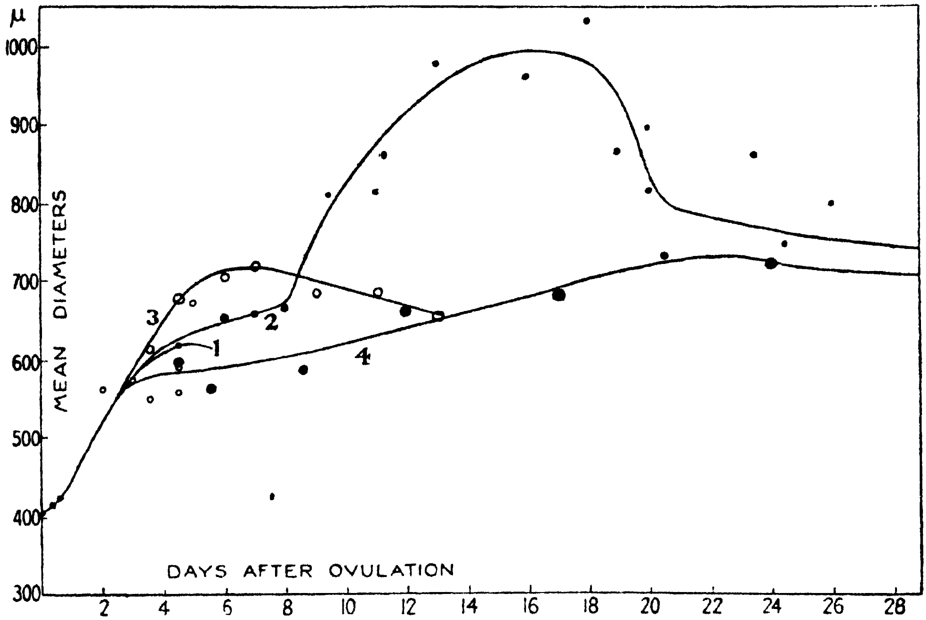
The sequence of histological changes, as the preceding account indicates, is essentially the same in all the corpora lutea of the mouse; there is a close

Table I.—Dimensions of the Lutein Cells at Different Stages of the Œstrous cycle.

Stage.	Mean diameters (μ).	
	Nuclei.	Cells.
Unmated—		
Follicular epithelium	5	Indefinite ; cytoplasm scanty
12 hours corpus luteum	5	Indefinite ; cytoplasm scanty
Metœstrous corpus luteum	6–7·7	10
Diœstrous corpus luteum	7–7·7	12
Pseudo-pregnant—		
3½ days p.c.	5–6	11–12
6 days p.c.	6–7	8–23 ; mean about 15
13 days p.c. (*post* ovulation)	5–6	Indefinite, shrunken
Pregnant—		
4½ days p.c.	5·5–6	7–12
8 days p.c.	6·5–7	10–18 ; mean 13
11 days p.c.	6·5–7	12–23 ; mean 18
13 days p.c.	6–8·5	17–30 ; mean 23
Post parturition—		
Preganey corpus luteum 1½ days *post partum*	6–6·5	Indefinite, about 9–12
Lactation—		
8½ days *post partum*	5–5·5	8–12
17 days *post partum*	6–7	15

Note.—The variations in the size of the nuclei and the varying irregular dimensions of the lutein cells make it possible to provide only approximate measurements. An apparent increase or decrease in the size of the nuclei or cells was checked by direct comparison of corresponding areas.

resemblance to those described in the rat by Long and Evans (10). The lutein cells enlarge and acquire fat and lipoid granulations, which undergo a steady or at first a fluctuating increase ; gradually the fat becomes very prominent and tends to fill the cells ; from being fine and regular, the granulation passes into a coarse, irregular condition. About the time the corpus luteum reaches this stage in the mouse the lutein cells and their nuclei shrink slightly, and the cytoplasm becomes deeper staining and the cell boundaries indistinct. The abundance of accumulated fat lasts only for a few days, after which it disappears more or less rapidly, leaving old corpora lutea fat-free (fig. 1) or sometimes with sparse irregular fat persisting for a considerable period (fig. 11). Old corpora lutea, which have recently lost the bulk of their fat but have undergone little shrinkage or displacement, are best identified as such by the appearance of their nuclei and cells. Detailed examination is particularly necessary in normal or experimental ovaries presenting difficulties of interpretation, owing to the absence of recent functional corpora lutea, as when ovulation failed to accompany the preceding œstrus, or during the prolonged lactation diœstrus.



Growth of corpora lutea in the mouse.

1.	Corpus luteum of ovulation			o.
2.	,,	,,	pregnancy	•.
3.	,,	,,	pseudo-pregnancy	○.
4.	,,	,,	lactation	●.

As regards the siderophil substance in the lutein cells referred to by Goormaghtigh (8) it is common to observe it in the corpora lutea during the first half of pregnancy or pseudo pregnancy; the lutein cells stain grey or almost black with iron hæmatoxylin, the stain being deepest in the outer-lying cells (*cf.* the transitional fatty corpora lutea). During the mid stages of pregnancy, and at about 6 days pseudo-pregnant (fig. 10), the majority of the lutein cells are light staining, but in patches they stain deeply. At 16 and 18 days pregnant the lutein cells are not siderophil, but at parturition the peripheral zone of the corpus luteum, where osmicated fat is accumulating, consists of these dark-staining cells. After the succeeding œstrus the grey siderophil cytoplasm is found throughout the corpus luteum.

Thus it appears that both an active and an inactive phase of the fatty secretory product, in association with the cell proteins, produces a siderophil substance in the cytoplasm, so that when present its significance must be considered in relation to other cell characteristics. The siderophil substance is undoubtedly associated with the changes undergone by the secretory product, but the histological findings must be compared with those obtained by other methods before further statements can be made.

Although there is a general similarity in the different mouse corpora lutea observed, it would be inaccurate to regard the histological changes as completely standardized. Corpora lutea of ovulation, for example, show individual

variations both in growth and in the rate of fat accumulation at the time of metœstrus, and the corpora lutea of pregnancy examined do not form a perfectly regular series. If the corpora lutea of different cycles are compared, it is noticeable that the initial growth is slowest and complete regression after cessation of function most gradual in the corpora which normally have the longest functional existence. Thus Long and Evans's statement (10) that distinctive characteristics are lacking for the corpus luteum of pregnancy during its first 9 or 10 days, when it resembles the corpus of ovulation, is not true for the mouse.

The details given in the text show that the corpus luteum of the mated cycle is distinguishable from that of ovulation at 2–3 days old, when the corpus luteum of ovulation has begun to accumulate fat. The smaller size of the nuclei and the greater regularity of the cells in the corpora of the mated cycle form other diagnostic characters. Cell enlargement and fat accumulation is more rapid in the corpus luteum in pseudo-pregnancy than after a fertile mating. The corpora lutea of the mouse, other than those of pregnancy, do not differ greatly in size (Table I), those of ovulation being only slightly smaller than those of pseudo-pregnancy and lactation. Clearly a different stimulus is operative during pregnancy, of which the effects become apparent about the tenth day after fertilization.

The view long advocated by Marshall (11) and others that the corpus luteum is a gland with an internal secretion is now generally held ; one of the effects assigned to it is the inhibition of œstrus. A discussion of the functional aspects of the corpus luteum, with special reference to the mouse, can be found in the recent literature (16–20, etc.). Here it is only relevant to consider the histological appearance of the corpus luteum at different phases of the œstrous cycle, including (*a*) when there is evidence to show that it is functional, and (*b*) when it is no longer functional as an œstrous inhibitor.

The activity of the corpus luteum in the mouse has been demonstrated during pseudo-pregnancy (19), pregnancy (16, 18) and lactation (17, 19). The evidence indicates that the corpus luteum of ovulation is not functional (20), and its apparent inactivity can be correlated to some extent with its histological appearance. Its lutein cells rapidly accumulate osmicated fat and lipoids, suggesting an inactive cell metabolism (8), and the vascular reticular system is less developed than in other corpora lutea of the mouse.

During pseudo-pregnancy the corpus luteum may be held to act as an œstrus inhibitor for 8 or 10 days, assuming the œstrus-producing stimulus to be active 2 days prior to the actual event (2) ; further, it has been shown

experimentally that the corpus luteum is functional in sensitizing the uterus, so that placentomata can be produced, up to 6 days after sterile copulation (19). A marked accumulation of osmicated fat is found in the lutein cells, however, by about the sixth day (fig. 11), and from then onwards the fat increases rapidly. In the series of corpora lutea examined, the mean diameter decreases after 7 days from copulation; but this may result from normal variation; actual cellular shrinkage is only perceptible just after the succeeding ovulation (fig. 1).

In comparison with the corpus luteum of pregnancy, just before parturition, the abundant fat in the lutein cells suggests a doubt as to whether the corpus luteum of pseudo-pregnancy is really functional after 6 or 7 days from copulation. It is possible, however, that the comparison is not a satisfactory one, since the corpus luteum at 18 days pregnant may not have entirely ceased to function; as the rate of production of its secretion diminishes, the effect may be swamped by an excess of œstrin, or of some other hormone.

The corpus luteum of lactation also presents difficulties of interpretation from a functional aspect; since, at least in some cases, fat accumulation and nuclear degeneration occur well before the next œstrus is due. It is noteworthy, however, that the evidence for the mouse of corpus luteum inhibition of œstrus during lactation relates only to the first 12 days *post partum* (17, 19), and similarly that placentoma formation is only recorded in the first 7 days *post partum* (19). It seems possible that smaller quantities of œstrin would have produced œstrus artificially during the third week, when, to judge from its microscopic structure, the corpus luteum of lactation is no longer in full functional activity. It has been pointed out elsewhere (15) that the inhibition of œstrus during lactation is dependent on the number of young suckling, so that if several factors are involved, variations in the structure of the corpus luteum of lactation at a given time after parturition, in different ovaries, would be by no means unexpected.

Two definite points seem to emerge from this study of fat and histological changes in the mouse corpus luteum: (1) Fat may be comparatively light in the lutein cells at the time when the corpus luteum ceases to be the controlling factor, as at 18 days pregnant, and (2) the fat-content may be heavy some days before any signs of the next œstrus appear, as in pseudo-pregnancy and lactation. It has long been held that a heavy accumulation of fat marks the decline of glandular activity, so it appears from the second statement that, although a functional corpus luteum inhibits œstrus yet it may decay without the next œstrus or ovulation occurring for a considerable time.

Other writers, including Long and Evans (10, p. 69), have already reached this conclusion on somewhat different grounds. Corner (6) finds rapid degeneration in the corpus luteum of ovulation in the sow about 15 days after œstrus, although the cycle lasts 18 to 23 days. Watrin (23, 24) and Horrenberger (9) show that the corpus luteum of ovulation in the human is histologically degenerate 10 or 12 days before the next ovulation is due. (Watrin notes also its inadequate vascularization, by comparison with the corpus luteum of gestation.)

There is evidence from the literature that, although ovulation is suspended during pregnancy, the corpora lutea do not always appear functional for the whole of the time, as they do in the mouse. They are conspicuously degenerate in the human. Watrin (23) describes the corpus luteum at term as fibrous, shrunken and almost fat-free—in fact in a late stage of decay. In correlation with this he notes that its operative removal when the embryo was only 2 months old did not terminate pregnancy. References to other records of such cases are cited elsewhere (20). Comparable results have been obtained on the guinea-pig, where the corpora lutea of pregnancy become very fatty some time before parturition (12); double ovariectomy is not in all cases followed by abortion (20). Thus the corpora lutea may be no longer essential to the maintenance of pregnancy, and histologically degenerate for a considerable time without a recurrence of œstrus taking place. Nadiejda (cited 22) finds histological regression in the corpus luteum of the rabbit from the fifteenth day of pregnancy onwards. Goormaghtigh (8), from a histological study of the corpus luteum of pregnancy in the dog, deduces a slowing-down in its functional activity at about the fifth week.

In other species, however, such as the spermophile (7), rat (20), and mouse (18), it has been definitely shown that the corpora lutea are throughout essential for the maintenance of pregnancy, and in these animals they are intact, or only in the initial stages of histological degeneration at the time of parturition

Thus there are indications that the duration of corpus luteum activity in relation to the cycle varies between different species, but with further experimental work there seems no reason to doubt that structure and function can be satisfactorily correlated.

Summary.

A description is given of the structure of the corpora lutea of ovulation, pseudo-pregnancy, pregnancy and lactation in the mouse, and the sequence of fat changes in these bodies.

The relation of histological structure to functional activity is discussed in

the light of available experimental evidence, and compared with conditions in other animals.

The work was done in the Department of Anatomy and Embryology, University College, London.

I wish to thank Prof. J. P. Hill, F.R.S., for his interest, and Dr. A. S. Parkes for supplying the mice from his colony, and for reading the paper.

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DESCRIPTION OF PLATES.

The microphotographs are the work of Mr. F. J. Pittock. With the exception of fig. 4, they show sections of material fixed in Flemming. Figs. 5, 7 and 10 show corpora lutea stained with iron hæmatoxylin, after the removal of osmicated fat by treatment with turpentine. The remaining sections are unstained. Figs. 1–3 are at a magnification of 48 diameters, and figs. 4–11 at a magnification of 110 diameters.

Abbreviations :—*c.o.*, corpus luteum of ovulation ; *c.lac.*, corpus luteum of lactation ; *c.pr.*, corpus luteum of pregnancy ; *c.psp.*, corpus luteum of pseudo-pregnancy ; *d.c.*, degenerated corpus luteum ; *foll.*, follicle ; *o.c.o.*, old corpus luteum of ovulation ; *o.c.pr.*, old corpus luteum of pregnancy ; *o.c.psp.*, old corpus luteum of pseudo-pregnancy ; *r.t.*, reticular tissue ; *str.*, ovarian stroma or interstitial tissue.

Plate 37.

Fig. 1.—Ovary at the end of pseudo-pregnancy, showing the corpus luteum about 8 hours old, formed at the recent ovulation (*d.c.o.*), and adjacent to it the old corpus luteum of pseudo-pregnancy containing abundance of osmicated fat. Note also old corpora lutea of ovulation (*o.c.o.*), and comparative absence of osmicated fat in the stroma.

Fig. 2.—Ovary from a 13 days' pregnant mouse ; the corpora lutea of pregnancy have begun to enlarge ; old corpora lutea have undergone rapid degeneration, and appear as shrunken fatty masses. Contrast with the old corpora lutea in figs. 1 and 11.

Fig. 3.—Ovary 4½ days after parturition, showing corpora lutea of pregnancy and lactation ; the degenerating corpora lutea of fig. 2 are here represented by conspicuous patches of fatty cells in the stroma.

Fig. 4.—From an ovary 12 days after parturition, showing a transitional corpus luteum of lactation ; the lipoids are coarser and more abundant at its exterior. Note also the corpus luteum of pregnancy, now much contracted, and the follicle about 380 μ in diameter, the largest type found in the ovary at this time. (Fixation, Ciaccio ; staining, Scharlach R.)

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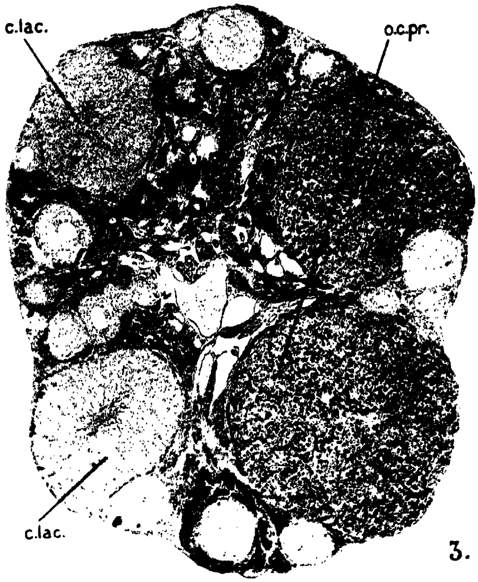

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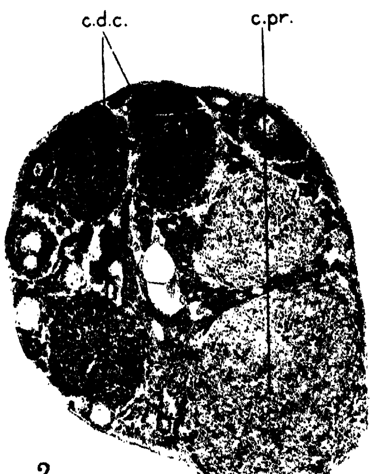

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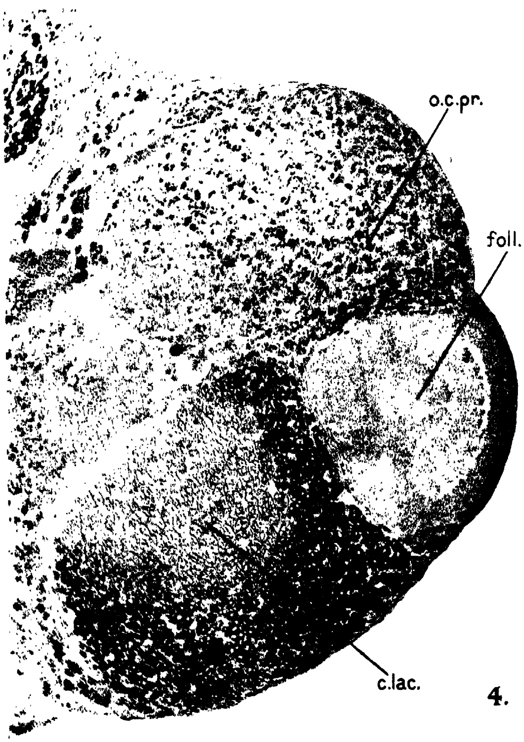

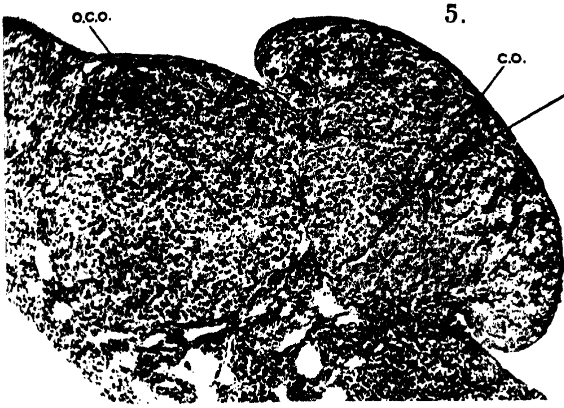
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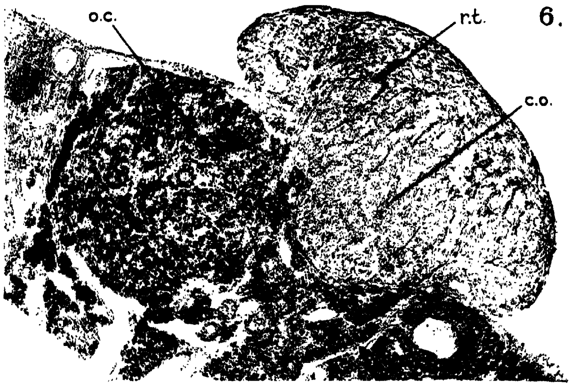
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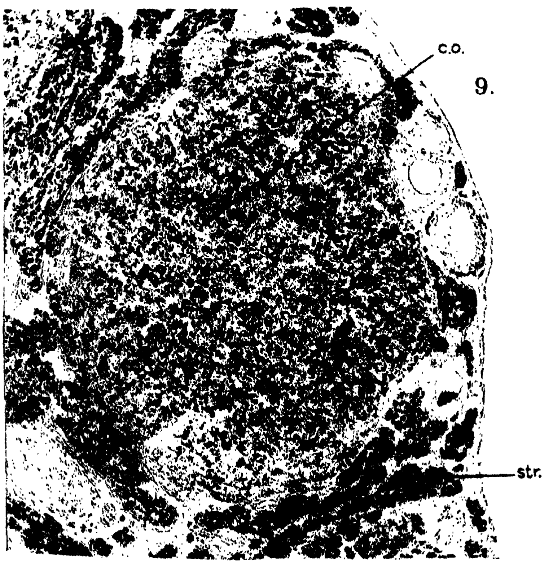
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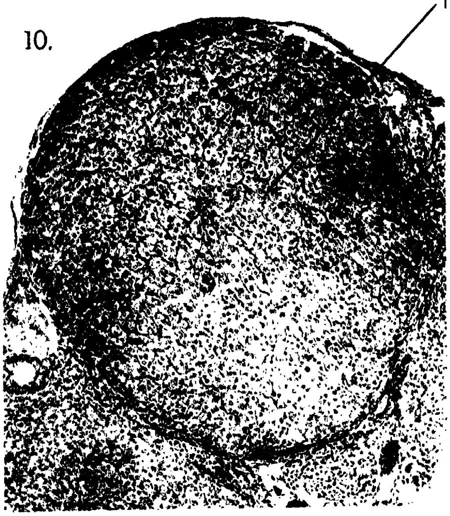

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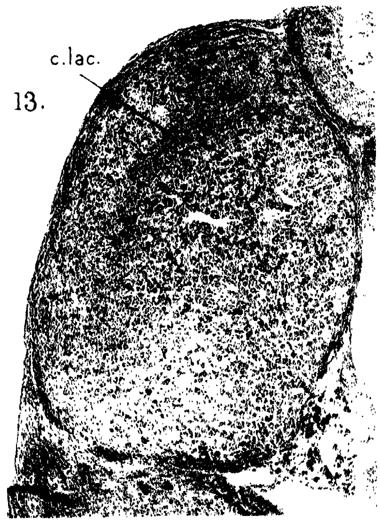

PLATE 38.

FIG. 5.—Corpus luteum of ovulation at metœstrus, showing the branching, dark-staining (siderophil) cells of the vascular tissue. The adjacent corpus luteum belongs to the last cycle.

FIG. 6.—The same corpora lutea as in fig. 5, from a different unstained section. Note that in the new corpus luteum there are only faint granules of osmicated fat near the inner edge. The corpus luteum of the last cycle is still very fatty.

FIG. 7.—Section through diœstrous corpus of ovulation; note the enlargement of the lutein cells compared with fig. 5.

FIG. 8.—Another section through the same corpus luteum as fig. 7; both the lutein and the endothelial cells contain fine granules of osmicated fat.

FIG. 9.—Corpus luteum of ovulation at the time of the next œstrus, showing accumulation of osmicated fat. This section was from an ovary of a young mouse in which the stroma was very fatty.

PLATE 39.

FIG. 10.—Corpus luteum of pseudo-pregnancy, 6 days after sterile copulation, showing nuclear and cell size, and dark-staining vascular tissue. On the right of the figure, some of the lutein cells have siderophil cytoplasm.

FIG. 11.—Corpus luteum of pseudo-pregnancy, from the same ovary as fig. 10, showing amount and distribution of the osmicated fat, which is more abundant in the outer zone. Adjacent to the corpus luteum of pseudo-pregnancy is an old corpus luteum of ovulation.

FIG. 12.—Corpus luteum of pregnancy from a mouse killed at the time of parturition, showing dimensions and fat granulation.

FIG. 13.—Corpus luteum of lactation at the end of the lactation diœstrus, 24 days after parturition. This ovary contained new corpora lutea, about 8 hours old. Note size and small amount of osmicated fat. Contrast with fig. 4, which shows abundance of lipoids 12 days after parturition.

612.813

The Effects of Injury on Mammalian Nerve Fibres.

By E. D. Adrian, F.R.S., Foulerton Professor of the Royal Society, the Physiological Laboratory, Cambridge.

(Received May 31, 1930.)

[Plates 40–46.]

When a nerve is removed from a frog and connected with an amplifier and recording system, occasional impulses appear if the fibres are allowed to dry and a short discharge can be produced by pinching or cutting, but if drying is prevented there is no sign of activity except during the actual infliction of an injury. Mammalian nerves give a very different picture. In a medium-sized nerve trunk from the cat or rabbit, set up in a moist, warm atmosphere, large and rapid fluctuations of potential are nearly always present and the disturbance may last for an hour or more in spite of repeated irrigation. Very small nerves may give a steady base line and in nerves prepared for recording motor or sensory discharges a disturbance, initially present, may subside during the exposure and manipulation, but in all mammalian experiments the danger of an unsteady background is increasingly present as the condition of the nerve is a closer and closer approach to the normal.

It is perhaps more surprising that an isolated frog's nerve should be inactive than that a mammalian nerve should be active. If a nerve trunk were dissected from its bed in a conscious subject, cut distally and suspended on electrodes, it is highly unlikely that there would be no pain from the exposed nerve except at the moment of division, and if it gave rise to persistent pain we should expect to find some evidence of persistent activity. An investigation of this persistent activity of mammalian nerves was therefore desirable for two reasons. It might lead to improvements in the technique of recording sensory and motor discharges in mammalian preparations and it might also throw some light on a problem which is still far from solution, that of the nervous mechanism responsible for pain.

The experiments have not yet advanced beyond the stage of a preliminary survey, but they show what sort of reactions may be expected from injured nerve fibres and the mechanism of these reactions has a theoretical as well as a practical interest since it appears to be closely related to that of the sense organs in general.

Method.

The animals used have been cats and rabbits, decapitated under chloroform anæsthesia and given artificial respiration. In some experiments the nerves were first examined without removal from the body, a short length being dissected free and slung on electrodes. The temperature and moisture were then controlled at first by warming the air with electric lamps and irrigating the nerve repeatedly, but at a later stage a large incubator was constructed to take the entire animal. More often the nerve was removed from the body and placed in a small water-jacketed incubator which served as a moist chamber. The floor of the chamber was flooded with water and the front was closed by a glass door with a small hole through which manipulations could be carried out. The temperature inside the chamber was maintained at 35–37° C. The nerve with its ends resting on small glass or vulcanite platforms was slung horizontally on two, or sometimes three, electrodes (Ag, AgCl, Ringer) of the usual U-tube form with cotton wool plugs upon which the nerve rested. As a precaution against drying a large pad of lint soaked in Ringer was arranged a few millimetres behind the nerve and both this and the nerve were irrigated with warm Ringer between observations. The temperature was shown by a small thermometer with its bulb touching the pad of lint behind the nerve.

The electrical activity of the nerve was recorded with a valve amplifier and a Matthews oscillograph, the particular arrangement being that described by Adrian and Umrath (1929). The amount of amplification is controlled by a tapped grid leak between the third and fourth valves. The total resistance is 1 megohm and the grid of the fourth valve can be connected to various fractions by a ten-way switch. With full amplification a potential difference of 5 microvolts at the input gives a deflection of 6 mm. on the recording surface and the base line is steady enough to show a potential of 2 microvolts when the resistance in the input circuit is 100,000 ohms. The period of the oscillograph reed is 5800 a second; this is high enough to give a close approximation to the true form of a monophasic nerve response at 37° C., but diphasic responses may be distorted when the distance between the leads is short. The distortion in the amplifying and recording system is checked from time to time by recording condenser discharges of known form and the make and break of a constant current through small non-inductive resistances. The system has also been tested for its ability to record a rapid series of impulses by sending into it a succession of discharges from a condenser in parallel with a neon lamp. At one stage it was found that an appearance of persistent negativity after an impulse was being produced by a faulty output valve, and some of the earlier records suffer from this distortion.

The amount of grid current passing through the first valve has always been less than 1 micro-ampere and the electrode circuit may be completely isolated from the grid by small condensers in either lead without affecting the activity of the nerve in any way.

The discharges can be viewed with a rotating mirror driven by an electric motor (*cf.*

Forbes, 1924*a*) and permanent records are made on bromide paper with two cameras, one for slow speeds (5–15 cm. per second) and the other with a rotating mirror giving an equivalent speed of 3·7 metres a second and taking a succession of records at intervals of 1 second. The spot of light from the oscillograph is split in half and an image is thrown on both camera slits, so that slow and fast records can be made simultaneously. The cameras are thrown in and out of action by plate clutches operated by Bowden wire controls. The electric disturbances are also converted into sound by a subsidiary amplifier and loud speaker, as described by Adrian and Bronk (1929).

Results.

The Three Types of Discharge.

Many different nerves have been examined and there are characteristic differences in the amount of activity they exhibit after removal from the body, but whatever the nerve and whatever the conditions under which the activity appears, the discharge of the individual fibres has always been found to conform to one of three main types. These are (1) a continuous and regular succession of impulses at a high frequency which changes very gradually and is rarely less than 150 a second (at 37° C.). (2) An irregular succession at a lower frequency (below 150 a second). (3) A grouped discharge, each group consisting of several impulses very closely spaced and the successive groups following one another at a very low frequency (10 a second or less). The main features of these three forms of discharge will be discussed in the present section, setting aside for the moment the conditions under which they appear.

The activity of the individual nerve fibres can be made out most clearly in the small cutaneous nerves, since in these the number of active fibres is often very small and may be reduced to one without much difficulty. The dorsal cutaneous nerves were generally used, for they are easily exposed and a dozen or more similar nerves can be obtained from one animal. Some preparations of these nerves show no kind of activity unless the fibres are allowed to dry or are stimulated by cutting, etc. Often, however, the smallest nerves are inactive when first set up, but after some minutes develop a grouped discharge (type 3) in one or more nerve fibres, whilst the larger nerves give discharges of the first and second type as soon as they are set up, and these subside gradually to reappear again if the nerve is allowed to cool slightly. By watching the discharge during the period of rise or decline it is often possible to select a stage at which only one or two nerve fibres are in action, and if the discharge persists in many fibres the nerve may be partially divided or the area from which the discharges originate may be progressively cut away. Fig. 1 (Plate 40) shows the three types of response which may appear in single

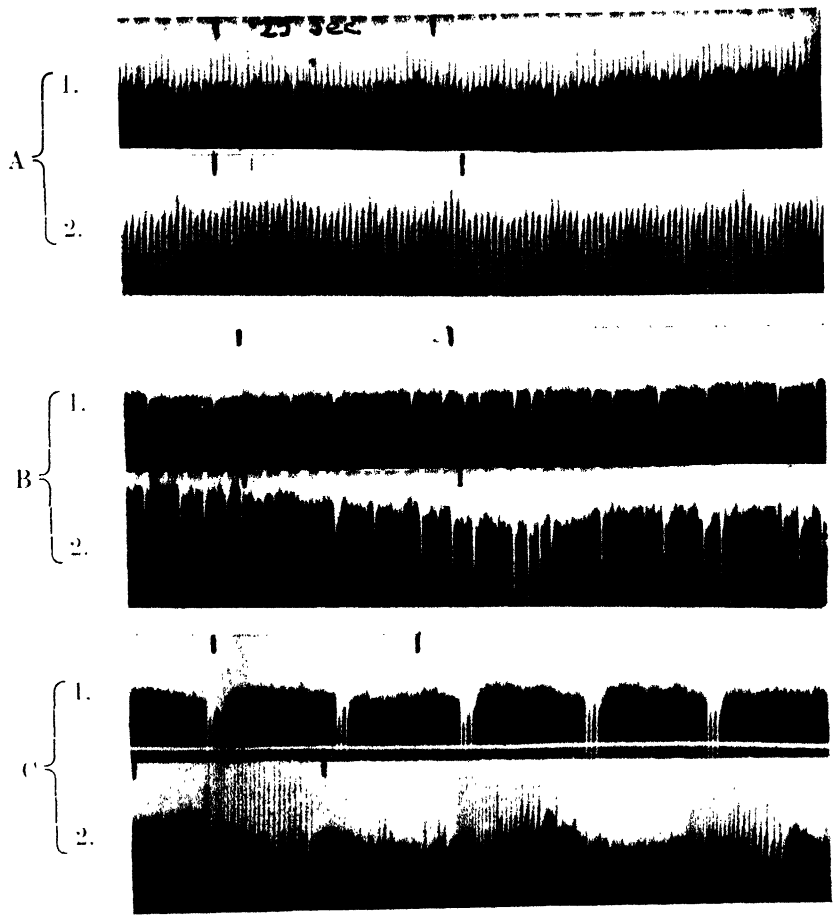

Fig. 1.—The three types of persistent discharge from isolated mammalian nerves. Records from dorsal cutaneous nerves of the cat, showing only one fibre in action. A—(1) Continuous discharge, frequency 144 a second; (2) continuous discharge, another nerve, frequency 156 a second. B—(1) Irregular discharge, average frequency 22 a second; (2) irregular discharge, another nerve, average frequency 25 a second. C—(1) Grouped discharge, 3 impulses in group, groups 6·6 a second; (2) grouped discharge, another nerve, 23–30 impulses in group, groups 3·4 a second.

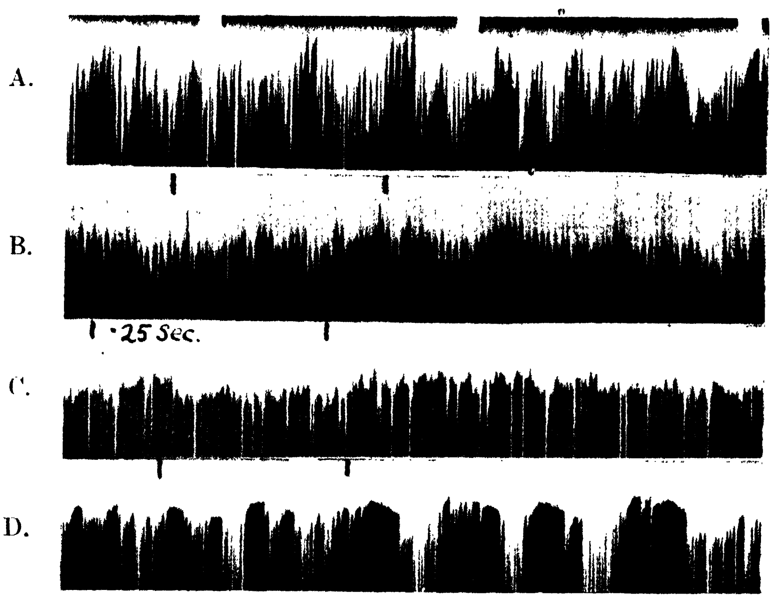

Fig. 2.—Composite discharges due to several fibres. A. Branch of intercostal nerve, many fibres in action. B, Cutaneous nerve, continuous discharge at 145 a second with one or more grouped discharges. C, Cutaneous nerve, continuous discharge at 175 a second with irregular discharge. D, Cutaneous nerve, multiple grouped discharges.

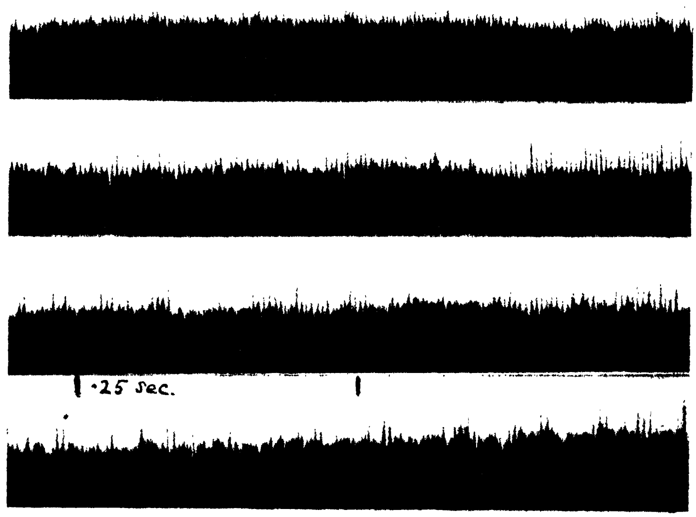

Fig. 3.—Dorsal cutaneous nerve. Continuous discharge (300 a second) changing into an irregular discharge of low frequency. The change was produced by lowering the temperature about 5° C.

fibres and examples of compcsite discharges of varying complexity are given in fig. 2 (Plate 41).

In the continuous and the grouped discharges (fig. 1, A and C) the orderly succession of impulses is the best evidence that we have to deal with the response of a single nerve fibre and further evidence is supplied by the fact that discharges of this kind can never be resolved into simpler components by further dissection. In the case of the irregular discharge (fig. 2, B) we have only the uniform size and shape of the impulses and the absence of any of the interference or summation which is likely to occur when several fibres are working independently. But even if we assume that more than one fibre is in action it is clear that the discharge in each must be irregular and of lower frequency than in the continuous type. The records given in fig. 1 are examples of a large number which have been obtained from nerves of all sizes and no other characteristic form of activity has been detected.

The continuous and irregular discharges are closely related. The former may slow down gradually and then as the frequency approaches the critical region of 200–150 a second occasional impulses are dropped out leaving periods where the rhythm still remains ; the gaps become more and more frequent and eventually all trace of rhythm disappears and the discharge has changed to the irregular type (*cf.* fig. 3, Plate 40). When this transition occurs it seems clear that the regular and irregular types of discharge are due to the same process operating at different intensities. But a continuous high-frequency discharge often remains in the region of 150 a second for a long period and then disappears abruptly without the irregular stage, and sometimes it may occur as a succession of active periods with a falling rhythm, each lasting ½ second or so and separated by periods of complete rest. This may be regarded as a transitional stage between the continuous and the grouped discharge, but a grouped discharge of the usual type seems to represent a distinct phase of activity fcr it does not develop into or out of the other types and it occurs under different conditions.

The grouped discharge often develops progressively in a nerve which was initially quiet. The first sign is an occasional impulse and then a succession of single impulses at a low frequency (5–20 a second). Within a few minutes most of the single impulses and later all of them are replaced by groups of two separated by about 5–7·5 σ (*cf.* figs. 4 and 5). The interval between the two impulses in the group becomes shorter and then a third impulse appears, at first in some and later in all the groups. The interval between the second and third impulses follows the same course as that between the first and second.

It begins at about 7·5 σ and becomes shorter and shorter until a fourth impulse appears again at an interval of about 7·5 σ from its predecessor. In this way the group may develop into as many as 8–10 impulses, the first two separated by 2 σ or less and the last two by 5–8 σ. During the evolution of the discharge the frequency at which the groups occur may decrease or may remain constant, but it is rarely greater than 15 or less than 5 a second. Mechanical interference with the region from which the discharge originates may produce an abrupt change in frequency, or it may stop the discharge or start a fresh series, but the frequency cannot be increased or decreased at will. It can, of course, be varied by a change of temperature.

A good example of the gradual development of a grouped discharge is shown in figs. 4 and 5 (Plate 42). Here the group frequency falls from 21 to 7·7 a second and each discharge changes from a single impulse to a group of eight. In the record the interval between the groups increases with the number of impulses in the group; this relation is found in most discharges but it is not an invariable rule, for several preparations have shown no change in the group frequency as the number in the group increases.

The Critical Interval in Relation to the Recovery Time of the Nerve Fibre.

The intervals between successive impulses in various grouped discharges are shown in fig. 6, and it will be seen that the longest interval has never exceeded 10 σ and is usually about 5 σ. It may, of course, exceed this value if the temperature is lowered.

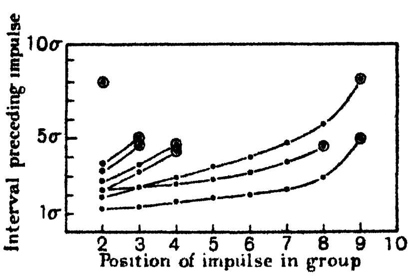

FIG. 6.—Spacing of impulses in various grouped discharges from different nerves. The final impulse in the group is marked with a ring. The interval preceding it varies from 4·3 to 8 σ.

If we compare these intervals with those occurring in the continuous type of discharge they are found to cover an almost identical range, for here too the maximum interval is in the neighbourhood of 7·5 σ. On the other hand in the irregular type the interval is always greater than 7·5 σ except in the occasional periods where a *distinct* rhythm appears.

Thus in every type of activity we find the impulses arranged in orderly sequence when the intervals between them are less than about 7·5 σ but the sequence is broken as soon as this interval is exceeded.

The existence of such a critical interval makes it almost certain that the

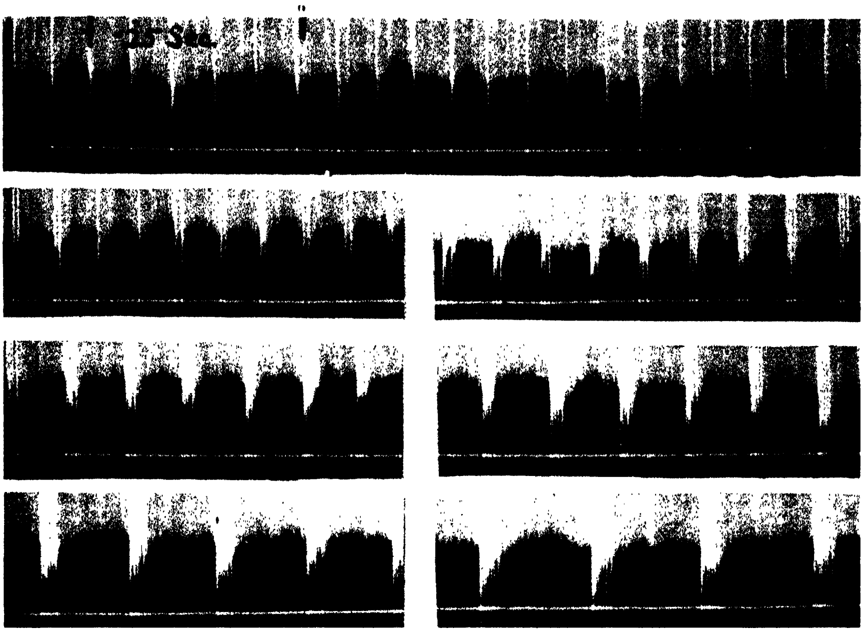

FIG. 4.—Gradual development of grouped discharge, dorsal cutaneous nerve of cat. Records made at intervals of 2–3 minutes.

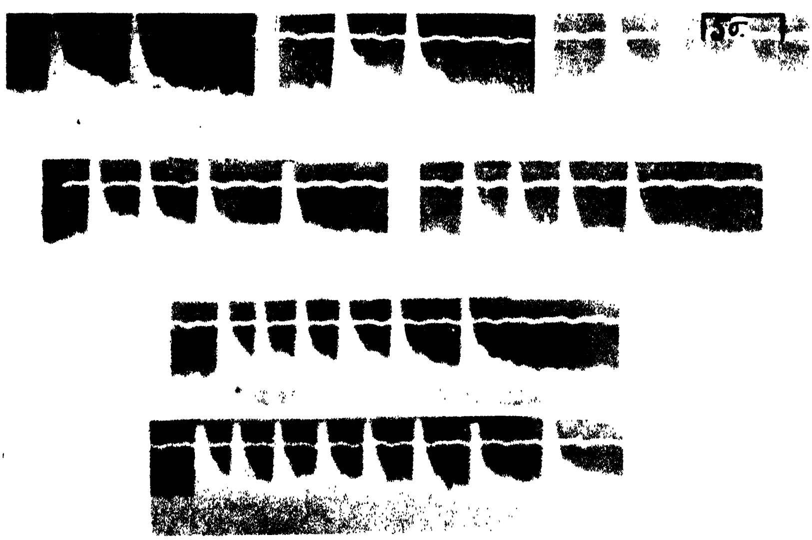

FIG. 5.—Records made with high-speed camera simultaneously with those in fig. 4, to show the spacing of impulses in each group. The interval between the last two impulses in the group fluctuates between 5·5 and 4–5 σ.

A.

B.

C.

D.

E.

F.

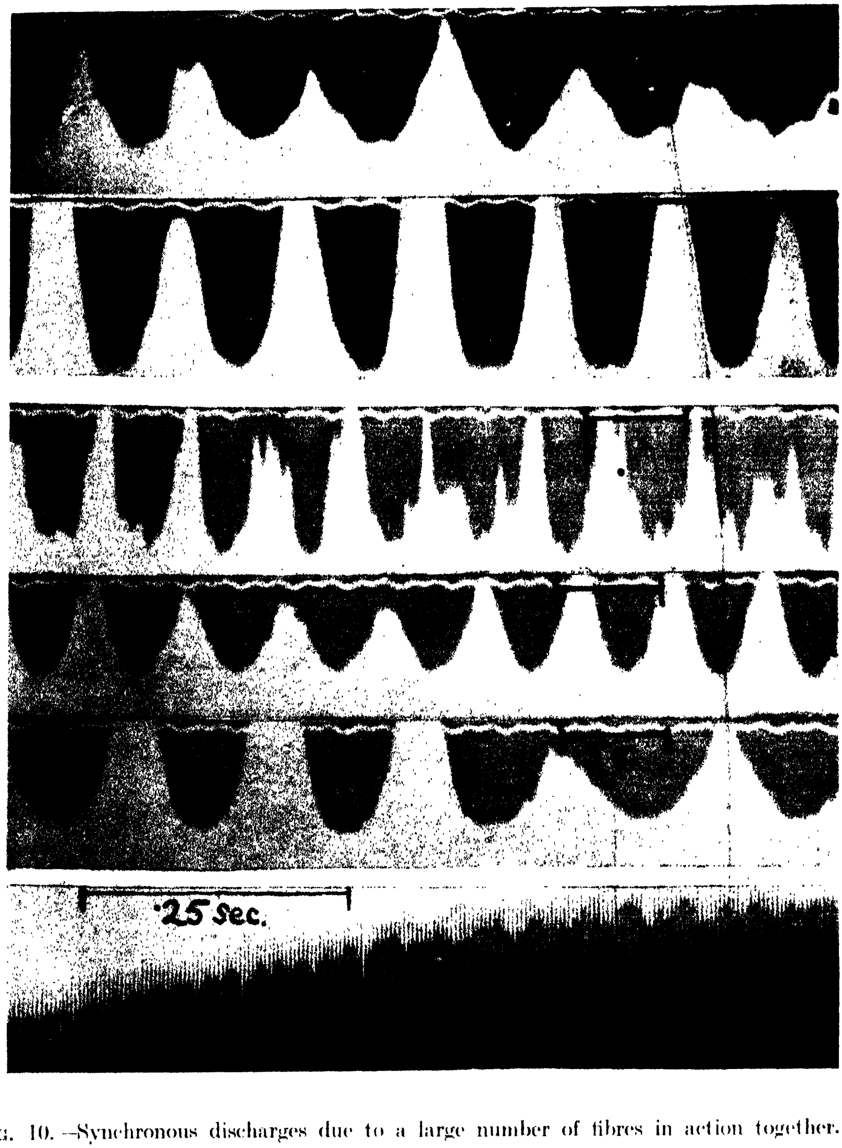

FIG. 10.—Synchronous discharges due to a large number of fibres in action together. A and B, long thoracic nerve (cat). Discharge became synchronised when the nerve was cooled slightly by opening door of incubator. The smaller excursions in B represent 0·35 millivolts. C and D, popliteal division of sciatic (cat). E, same nerve, effect of cooling by opening incubator. F, peroneal nerve, a few seconds after a fresh cut, largest excursions represent 0·53 millivolt.

detailed structure of the discharge is simply dependent on the period of recovery in the nerve fibre. The passage of an impulse in a mammalian nerve sets up a refractory state which is absolute for about $0 \cdot 7\,\sigma$, and this is succeeded by the relative refractory period during which the fibre gradually regains its initial excitability. We cannot say precisely how long must elapse before recovery is complete in the particular fibres which give the persistent discharge, but the existing curves for motor fibres (Adrian and Olmsted, 1922) and Erlanger, Bishop and Gasser's measurements (1926) of the absolute refractory period in different kinds of fibre show that we shall not be far out in taking the total recovery time as somewhere between 5 and $10\,\sigma$. Thus for a period of 5 to $10\,\sigma$ after the passage of an impulse the excitability of the fibre is increasing from zero, but after this its value remains constant apart from the slight changes due to the supernormal phase, etc. If instead of the excitability we take the strength of stimulus needed to set up an impulse we have a recovery curve of the form shown in fig. 7. This is a transcription of the curve determined by Adrian and Olmsted (1922) for the motor fibres of the cat's peroneal nerve and it shows a somewhat slower recovery rate than usual. In practice such a curve is constructed by applying a momentary stimulus to the nerve at different stages of recovery and finding how strong it must be in order to set up an impulse, but if instead of a momentary stimulus we could use one which maintained a constant excitation, we should expect that a fresh impulse would be set up whenever the recovery from the previous impulse was sufficiently advanced. Thus a constant stimulus three times the threshold strength would set up a series of impulses at a frequency of 500 a second (a^1, a^2, a^3, fig. 7) because the fibre takes 1/500 second for its excitability to rise to the level at which this stimulus becomes effective,* a stimulus of twice threshold strength would give a series with a frequency of 333 a second (b^1, b^2, b^3) and so on. But in this nerve no kind of constant stimulus could give a regular series of impulses at a lower frequency than 100 a second because

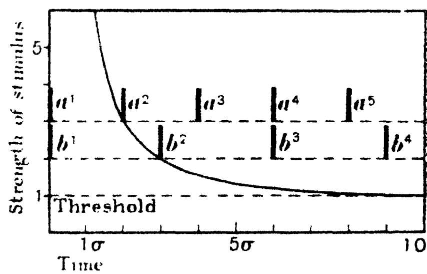

FIG. 7.—Recovery curve of motor fibres in cat's peroneal nerve (Adrian and Olmsted), showing the repeated discharges which would be produced if the excitation remained constant.

* It is assumed, for simplicity, that the rate of recovery is not altered by the repeated activity of the fibre.

there is no longer any progressive increase of excitability after an interval of 10 σ. A constant stimulus just below the threshold strength might set up an occasional impulse owing to slight fluctuations in the excitability of the fibre and it would give an occasional group of several impulses regularly spaced whenever the excitability rose sufficiently.

It follows that the response to a steady excitation of gradually decreasing intensity would be an orderly series of impulses repeated with a frequency which would fall gradually to about 150 a second and the response would then cease or become irregular (fig. 8, A). In the same way an excitation which

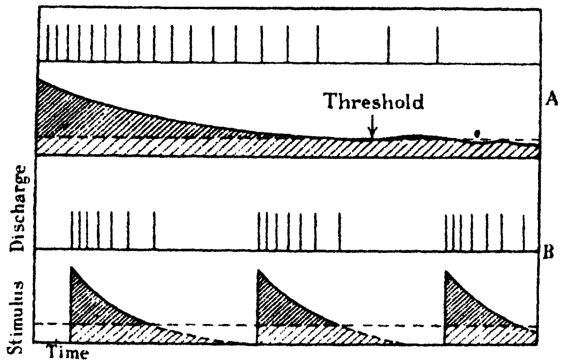

Fig. 8.—Production of continuous discharge (A) and grouped discharge (B) by prolonged excitatory periods.

decreases more rapidly would give a small group of impulses spaced at increasing intervals and the last two would be about 1/150 second apart. This is shown in fig. 8, B, which indicates how a grouped discharge would develop as the result of repeated periods of excitation.

It might be objected that the impulses in a group can scarcely be travelling in an incompletely recovered nerve fibre since the later impulses show so little reduction in size compared with the first. The records given in fig. 9 (Plate 46) make it clear that there is some reduction in the second action potential when the interval between it and the first is small enough. But there is really no reason to expect a greater reduction, for hitherto all experiments concerned with the point have measured the total electric effect of all the fibres in a nerve and the results have been complicated by the differences in recovery rate in different fibres (*cf.* Zotterman, 1928, Amberson, 1929). The present records suggest that the nerve fibre's capacity to develop the action potential returns at least as rapidly as the excitability and possibly more rapidly, and in this they agree

with results obtained by Matthews (*unpublished*) on single fibres of the frog's nerve activated by end organ stimulation.

We may conclude that the detailed arrangement of the continuous and grouped discharges is due to the periodicity imposed by the recovery process of the nerve fibre, and that the excitation remains relatively constant over periods which are long compared with the total recovery time of the fibre.

This conception makes it possible to account for a remarkable change which is sometimes observed in the discharge of the more active nerves.

Synchronous Discharges.

When a fair-sized nerve like the phrenic or long thoracic is removed from the body and placed on the electrodes, there is usually a persistent discharge which appears as an irregular succession of potential changes of high frequency and medium amplitude (*e.g.* 0·05 mv.). Many nerve fibres must be taking part in the discharge but they are working independently and the individual rhythms can only be seen when the number of fibres in action has been much reduced. On many occasions, however, the confused noise produced by transforming the fluctuations into sound has changed suddenly or gradually into a definite musical note of considerable volume, and an optical record shows that the discharge has now become a regular succession of very large waves with a frequency between 150 and 600 a second. Several examples of these rhythmic discharges are given in fig. 10 (Plate 43). In comparing them with previous records it should be noted that the changes of potential are from 10 to 30 times as large as those which were regarded as the product of single nerve fibres, but the frequency range is the same, for it never falls below 150 a second unless the temperature is lowered. The form of the potential change differs considerably from that due to a single fibre and is often a near approach to a sine curve, but the records may show beats and, indeed, there is every gradation between complete irregularity and a completely regular wave form. The form is sometimes so regular that it suggests an artifact, but this possibility can be excluded by a number of controls. One of the most convincing is the change in rhythm produced by a fall of temperature (fig. 10, E.).

A large number of units discharging independently yet all at the same frequency would give composite effect in which this frequency could be detected, but the amplitude of the oscillations would be small unless there were some tendency for many of the units to discharge at the same moment. Thus the units must be synchronised with one another and we have to explain how this can take place.

The most reasonable explanation seems to be that an active fibre can cause a slight momentary increase in the stimulus to other fibres and that it can do so owing to the action current which it produces. The intact fibres in a nerve trunk can certainly become active without exciting their neighbours, but all that is necessary in the present case is that a very slight stimulating effect should occur. If the fibres are discharging because they are exposed to a constant stimulus, and if the periods of activity in one fibre can cause a slight periodic increase in the stimulus to its neighbour, there will be a tendency for the two to discharge in unison. Very little synchronisation could occur if the different fibres were all responding at different rates, but a group of fibres with the same frequency might come to dominate a larger and larger area round provided that the frequency was sufficiently high for the group to act as a pace maker to the rest. Thus the conditions favouring synchronisation would be a large number of active fibres and an intense and fairly uniform degree of excitation. An explanation on these lines would hold good whatever the nature of the stimulating effect produced by activity, but the conditions under which the effect appears are strongly in favour of electric forces as the synchronising agent. A slight readjustment of the end of the nerve on its glass platform will often cause a sudden change from an asynchronous to a synchronous discharge, and the determining factor appears to be the thickness of the layer of fluid in contact with the cut end of the nerve and its exact position in the layer. This makes it unlikely that the effect is due to something liberated at each impulse and reaching the neighbouring fibres by diffusion, but it becomes quite intelligible if the effect depends on the precise distribution of current at the end of the nerve.

Other examples of the synchronisation of rhythmically active cells by their electric effects have been described by Lillie (1914), but the present case is interesting because of its likeness to the synchronous discharges produced by groups of nerve cells (Adrian and R. Matthews, 1928). Here too the essential factor appears to be a uniform and intense stimulation, and the same kind of explanation could be applied, namely, that the rhythmically active cell can influence its neighbour because it causes slight fluctuations in the intensity of an otherwise steady stimulus. The "double impulses" which Matthews (1929) has recorded in frogs' nerve may be due to a synchronisation of two fibres by the same mechanism.

Nature of the Stimulus causing Persistent Discharges.

(1) *Effects of Injury.*—We have supposed that the sequence of impulses in these discharges is governed by the recovery time of the nerve fibre, and that

the stimulus which arouses them is something which produces relatively long periods of excitation. Since the nerves have all been cut in their removal from the animal it is natural to associate the stimulus with something developed by the injured region.

Although it is well known that injury has a brief stimulating effect on motor nerve fibres, it could not be assumed without proof that it was the cause of these persistent discharges. In spite of the characteristic time relations it was possible that they arose not from the cut ends of fibres but from specific end organs in the nerve trunk excited by the abnormal conditions. There is no doubt, however, that the cutting of the nerve fibres marks the beginning of most discharges of the continuous or irregular type. A nerve trunk which is merely raised from its bed and slung on tripolar electrodes* may show some activity, but this is greatly increased as soon as a cut is made, and the initial activity is almost certainly due to the cutting of small branches when the nerve is raised (see p. 612). If the discharge declines some time after the nerve has been isolated it is usually revived by a fresh cut. Again the discharge can be abolished or greatly reduced by dipping the cut ends of the nerve in 10 per cent. novocain solution, and it will return again when the nerve is cut through at a point which has not been exposed to the drug.

The grouped discharge does not often appear as the immediate result of nerve section and it was thought for some time that it was a specific reaction due to endings near the nerve trunk. As a rule it arises from some point where a small blood vessel remains attached to the side of the nerve and it ceases when this is cut away. But it can also arise from the cut end of a nerve trunk and must then be regarded as a response of certain nerve fibres to injury. It is quite possible, however, that the mere exposure of susceptible fibres to abnormal conditions may be a sufficient stimulus without any gross destruction of the fibres. Drying, heating or crushing have much the same effects as cutting and no doubt less drastic changes may account for some of the discharges.

The stimulating effect of an injury is generally supposed to be due to the breakdown of the polarised membrane enclosing the nerve fibre. If the wave of activity which constitutes the impulse is due to a wave of depolarisation which propagates itself down the fibre, it should be possible to start an impulse by any process which leads to local depolarisation, and if we accept the view

* When a nerve is undivided, two earthed electrodes are used with the input electrode between them. This guards the input from electric changes occurring in other parts of the body.

that the spread of depolarisation in an intact fibre is caused by the action current, we may regard the injury current as the cause of the wave which starts from a cut region. But the injured region is permanently depolarised; in these nerves the injury potential averages about 10 mv. and remains very near its initial value for an hour or more. On the other hand the stimulating effect declines very rapidly in most fibres, for the number which keep up a persistent discharge is never more than a small fraction of the whole nerve. If the injury current is indeed the stimulus, there must be some opposing change which makes it rapidly ineffective in the majority of the fibres.

The decline in effectiveness of a constant stimulus is, of course, an old story. A weak constant current applied to a nerve produces a brief muscular twitch and not a tetanus, and we should not expect the constant injury current to do more as far as the motor fibres are concerned. This rapid failure of a constant stimulus is usually put down to some opposing reaction in the nerve fibre or its surroundings. Bishop, for instance, has recently suggested (Bishop, 1928) that it is due to the gradual polarisation of a structure which is in series with the nerve membrane and so in control of the current which reaches it. But whatever the opposing reaction may be, it is reasonable to suppose that its development may depend on the nature and conditions of the fibre, and on this view the variations as between one fibre and another and the fluctuations in the discharge of individual fibres would be due to variations, not in the injury current but in the reaction which opposes it.

No direct proof can be given to show that the stimulating effect of an injury is due to the depolarisation and to the current which it produces, but a great deal of indirect evidence has accumulated in the past starting from the experiments which demonstrated the injury current by the use of Matteucci's "Rheoscopic Frog." Lillie (1929) has recently strengthened the evidence, as far as these persistent discharges are concerned, by showing that in his iron wire model a region which is kept permanently active will set up a rhythmic succession of waves in the rest of the wire. In fact the present view is a necessary consequence of the membrane hypothesis, and until this has been found wanting there is no need to consider other possibilities.

Condition of Injured Fibres.

The stimulating effect of an injury will naturally be modified by various conditions which affect the uninjured part of the nerve fibre. For instance, a slight fall of temperature will often increase or start the discharge and it may be possible to make it appear and disappear at will by opening and shutting

the door of the incubator. More rarely a slight fall of temperature stops the discharge ; a considerable fall (to 20° C.) nearly always reduces it very greatly.* The effect of cooling probably results from some change in the physical condition of the myelin sheath, a change which may show itself in the slightly increased opacity of the nerve.

Another factor of some interest is the amount of irrigation to which the nerve has been subjected. In the larger nerves there is so much persistent activity that the effect of irrigation cannot be detected, but in the small cutaneous nerves repeated flooding with Ringer's fluid during dissection increases the activity. The effect is seen most clearly in connection with the grouped discharge, for this will often develop gradually when the nerve is placed in Ringer and will decline again if it is placed in serum or put back under the skin of the animal. In several preparations a grouped discharge has been evoked and suppressed in this way four or five times in succession over a period of an hour. Various changes were made in the composition of the Ringer without affecting the result, and it appears that the effect is due not to any abnormal salt concentration but to the gradual loss of something which is washed away from the fibre by Ringer and replaced slowly in serum. The nature of this substance has not yet been determined.

It may be noted that a change in the amount of calcium in the perfusing fluid has no obvious effect on the grouped discharge until the concentration reaches 0·1 per cent. Irrigation with a fluid containing 0·1 per cent. or more $CaCl_2$ slows the rhythm and finally abolishes the discharge, but irrigation with pure NaCl solution instead of Ringer does not alter it appreciably. This result is not surprising, for nerve fibres are much less susceptible to ionic changes than are nerve endings or muscles.

The gradual development of the grouped discharge during irrigation might be explained by supposing that the injury current is at first prevented from stimulating by a steady opposing force or barrier, and that the loss of some constituent weakens the barrier in such a way that it breaks down and reforms periodically, allowing the current to act for longer and longer periods on the excitable part of the nerve fibre. An alternative explanation is to suppose that a region of permanent depolarisation is not essential, but that the surface

* It might seem that the absence of persistent activity in the nerves of the frog could be explained by the lower temperature, but a frog's sciatic gives only an occasional impulse at 37° C. under exactly comparable conditions. The nerves of the tortoise are equally quiet. The larger nerves of the pigeon show some activity, but grouped discharges have not been seen. The nerves of guinea pigs and rats behave like those of the cat.

layers of the fibre are brought into a state in which they tend to become depolarised periodically, each period being brought to an end by forces of the same kind as those which operate in the normal fibre to restore the inactive state after an impulse. The same possibility exists in regard to the irregular type of discharge. The low frequencies with which this may occur suggest that we are dealing not so much with random fluctuations in the strength of the stimulus, as with a slow building up of the excitatory condition and its dissipation at the discharge of each impulse. But injury may disturb so many of the properties of the fibre that it would be useless to consider the mechanism of these discharges in greater detail.

The variability of the different nerves and the impossibility of removing them under strictly comparable conditions makes it difficult to compare one animal with another, but there is very little doubt that the nerves of some animals are much more active after injury than those of other animals of the same species. On the whole great persistent activity in the cutaneous nerves seems to be associated with vascular dilation in the subcutaneous tissues, but there have been many exceptions to the rule.

One other factor may be mentioned as affecting the results of injury and this is the presence of dense layers of tissue near the injured region of the fibre. It has been stated already that the very small cutaneous nerves are often inactive when isolated and may remain so, whilst the larger nerve trunks are invariably active. This is no doubt due in part to the presence in the larger nerves of a greater proportion of susceptible fibres, but this is not the whole explanation, for the difference between large and small nerves can be shown in the case of motor discharges. When the sciatic or main peroneal trunk is cut through in a spinal cat, the leg muscles give a contraction which does not subside completely for several seconds. A good instance of this has been put on record by Forbes (1924*b*). But as a rule cutting the small branches which supply each muscle gives no more than a brief twitch. The difference can be detected most easily by recording the electric response in the muscle. It seems to depend mainly on the thick sheath of the larger trunks, for if this is stripped away for a short distance in the middle of the nerve (fig. 11) a cut above or below the stripped region will give a discharge of several seconds' duration, but a cut through the region will give only one or two responses. Records showing this are given in fig. 12 (Plate 44). Stripping the sheath has the same effect on the persistent discharges in the nerve, for these are abolished or greatly reduced by removal of the sheath for 1 cm. or so from either end.

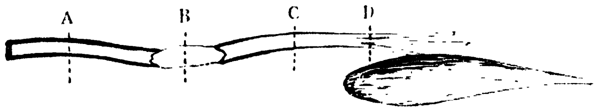

Fig. 11.—Effect of nerve sheath on discharge in motor fibres. A cut at A or C gives a discharge lasting a second or more, but a cut at B or D gives only three or four impulses.

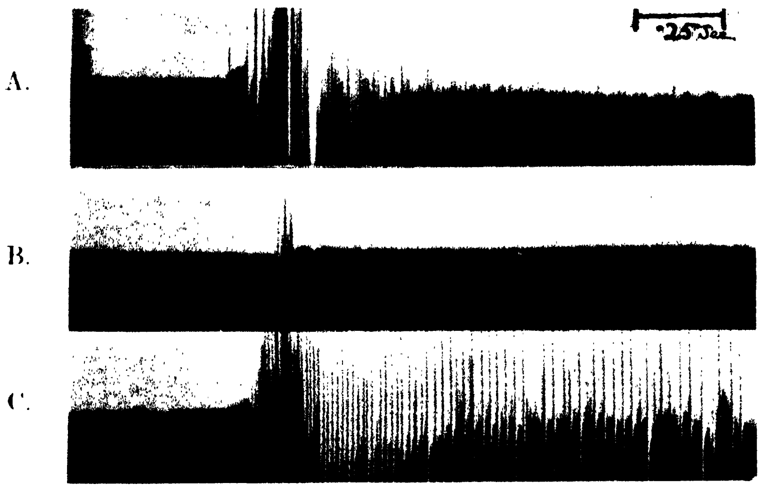

Fig. 12. Electric response of tibialis anticus muscle on cutting peroneal nerve at points marked A, B and C in fig. 11. Responses recorded with concentric needle electrode in muscle.

A.

B.

C.

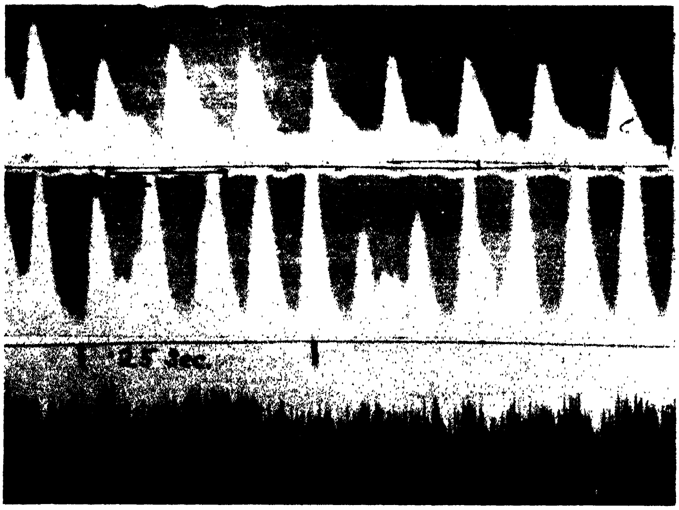

Fig. 13.—Continuous discharges produced by the action of $BaCl_2$ crystals on small nerves. A, single series of impulses, excursions = 20 microvolts. B, discharge in several fibres, partial synchronisation. C, synchronous discharge (incomplete) in many fibres, excursions = 0·5 millivolt.

A.

B.

C.

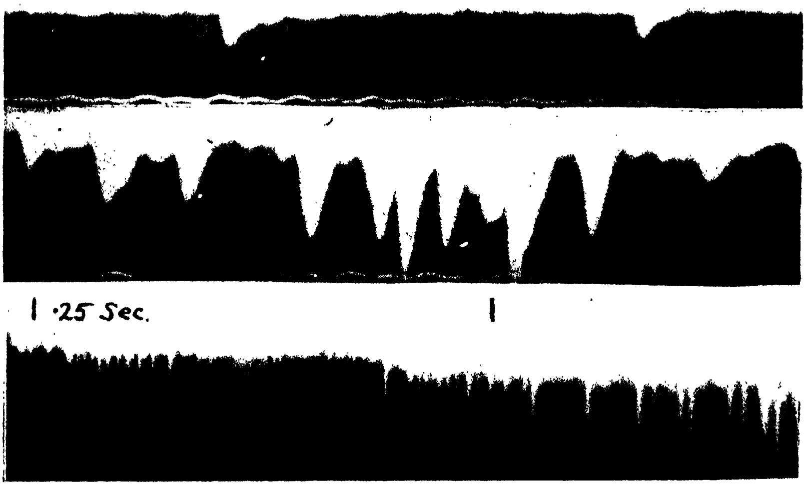

Fig. 14.—Comparison of spontaneous discharges with those due to touching hairs (A and B), and stretching muscle (C). A and B, skin and cutaneous nerve preparation (cat). A shows persistent discharge (irregular). B shows the larger impulses produced by touching the hairs with a glass rod. C, record from uppermost root of phrenic nerve (cat). Cut end treated with novocain. A grouped discharge occurs twice in the record and half way through a series of large impulses begins. These are due to the deflation of the lungs and the stretching of the diaphragm by upward pressure of the viscera.

It is unlikely that this effeot is due to anything liberated from the injured sheath or connective tissue, for injured tissue placed in contact with the damaged region will not start a discharge and repeated washing with Ringer will not stop one which is in progress. Nor is it likely that a thick sheath modifies the injury by causing more crushing of the nerve fibres, for crushing a small nerve with artery forceps does not cause an appreciably longer discharge than cutting. A more likely explanation is that the structures surrounding the fibre will influence the path taken by the injury current on its way from the intact to the damaged region. Bishop, Erlanger and Gassen (1926) have shown that the nerve sheath is a polarisable structure and has the effect of surrounding the nerve with an envelope of high transverse resistance. The presence of such a sheath round the intact part of the fibre will obviously restrict the path open to the injury current. This must pass from the intact to the damaged surface mainly in the very thin layer of fluid inside the sheath ; the resistance of the circuit will be higher than it would be if the sheath were removed and a smaller current will flow. It might seem from this that the stimulating effect would be diminished instead of increased, but a consideration of the circuits involved shows that with the restricted pathway the resistance will rise much more rapidly as the distance along the nerve is increased. Thus with the sheath there will be a much steeper gradient in the depolarising effect due to the injured region, and on general grounds we should expect that a steeper gradient in the depolarising effect would be more likely to disturb the balance between neighbouring parts of the fibre. But it must be admitted that if the sheath had been found to decrease the stimulating effect instead of increasing it, the restriction of the total current by the sheath might have seemed an equally plausible explanation.

The results of this section may be summarised as follows. The persistent discharges start from an injured region in the nerve fibre and are probably due to the depolarising effect which it exerts on the uninjured regions. The effect is increased when the fibre is surrounded by dense structures such as a thick nerve sheath and it is also increased when the fibre is brought into an abnormal condition by irrigation, etc.

Nature of Fibres concerned in the Discharge.

The fibres which are most liable to give persistent discharges appear to be sensory fibres of small diameter. That the motor fibres do not normally react in this way is shown by the brief contraction produced by cutting a motor nerve, for persistent discharges may then be detected in the nerve long after

the muscle has become completely inactive. It is true that the failure of the muscle might be due to nerve ending fatigue if the motor fibres were discharging continuously at a very high rate, but if they were doing so a second cut would be unlikely to produce a contraction of the same duration as the first, and it is found that repeated discharges in the muscle, each lasting 2–3 seconds, may be produced by the repeated division of a large nerve trunk.

The fact that the muscle may remain active for several seconds when the nerve to it is cut shows that the difference between motor and sensory fibres is one of degree only, and the absence of any sharp distinction between the different types of nerve fibre is also shown by the effects of various irritant substances. If a crystal of sodium, calcium or barium chloride is placed in contact with a small cutaneous nerve, first one fibre and then another begins a discharge of the continuous type with a frequency rising very rapidly to 500–800 a second, until so many fibres are in action that it is impossible to make out the individual rhythms. At the height of the discharge the impulses may become synchronised. Examples of the discharge induced by $BaCl_2$ are given in fig. 13 (Plate 45). In this case the effect is not confined to one class of fibre, for the motor fibres are brought into a condition in which they too give a persistent discharge. A crystal of $BaCl_2$ placed in contact with the cut end of the peroneal nerve will produce a steady contraction in the tibial muscles lasting 10 minutes or more, though it is true that the electric response of the muscle fibres is usually irregular and below 150 a second in frequency.

Under normal conditions, however, the motor fibres give only a brief discharge on cutting, and the efferent sympathetic fibres may be ruled out, since there is no sign of erection of hairs or other effects of sympathetic excitation when a cutaneous nerve has been cut. Thus the effect seems to be largely confined to sensory fibres. The evidence for their small diameter rests in part on the small size of the monophasic action potentials as compared with those produced in the same nerve by stimulating various end organs. If a strip of skin is removed from the back of the cat with the cutaneous nerve attached, the preparation will remain alive in the incubator for upwards of an hour and impulses will pass up the nerve whenever the hairs are moved. If some kind of persistent discharge is also passing up the nerve from the cut ends of fibres the two sets of impulses can be compared. Neither the action potentials of the persistent discharge nor those due to touching a hair are all of the same size, but the largest of the hair impulses are invariably greater than those which form the persistent discharge and are usually from three to five times as great. Typical records are given in fig. 14, A and B (Plate 45). These are

made on a rapidly moving surface to avoid the danger of mistaking two overlapping responses in different nerve fibres for a single large response. It is possible that the large waves which have the time relations of a single response are due to two smaller responses occurring simultaneously, though their frequent appearances makes this improbable. This source of error is avoided when we lead from a nerve supplying a muscle and compare the persistent discharges with those produced by stretching the muscle, for here the impulses from the tension receptors are of much more uniform size and can often be identified by their regular rhythm. Fig. 14, C, shows a record of this kind made from the uppermost root of the phrenic nerve. A typical grouped discharge occurs at intervals and half way through the record a series of large impulses begins. These are due to the stretching of the diaphragm by the viscera when the lungs are deflated.

Gasser and Erlanger (1927) have made their reconstructions of composite action potential curves by assuming (*a*) that the rate of conduction in a fibre is proportional to its diameter and (*b*) that the action potential which it will produce at the electrodes is proportional to its cross sectional area. On the latter assumption the diameter of the fibres giving the persistent discharge would be about half that of the largest fibres from the hairs and muscle receptors. But Matthews (1929) has recorded impulses in nerve fibres from the frog's skin and muscle and his figures show the rate of conduction and the size of the action potential varying in the same proportion, both being about 20 per cent. less in the cutaneous than in the muscle fibres. In view of this discrepancy we cannot use the size of the action potential to give more than a very rough estimate of the size of the fibres concerned, and all that can be said is that most of them are smaller than the large sensory fibres. Measurements of their conduction rate have been attempted and the figures obtained range from 20 to 45 metres a second, but the probable error is so large that the only conclusion it is safe to draw from them is that the fibres do not belong to the very slow C group of Erlanger and Gasser (1930).

Many of the fibres are concerned in the innervation of blood vessels and connective tissue. A good example of this occurs in the case of the phrenic nerve. This always shows great activity after removal from the chest—in one phrenic a group of fibres gave a synchronous discharge at 200–300 a second for 20 minutes, a total of some 300,000 impulses in each fibre. The potential changes which occur when the discharge is fully synchronised may be as large as 1 millivolt, and as the injury potential of the whole nerve is 8–12 millivolts we may assume very roughly that about one-tenth of the 700 fibres in the nerve

are engaged in the discharge. But a considerable part of the discharge arises not from the cut ends of the nerve but from its whole length, for the discharge is reduced but never completely abolished by treating the cut ends with novocain, and the form of the diphasic responses with various positions of the electrodes shows that the impulses are starting from scattered points along the course of the nerve. Since the cat's phrenic has no large branches in the thorax, the origin of this discharge was puzzling, but the phrenics are known to supply fibres to the pleura and pericardium and they are in close relation to the large blood vessels. To determine how many fibres actually leave the phrenic in its course through the thorax, several nerves were fixed in osmic acid and sections were cut and photographed so that the number of fibres at different levels could be counted. The results are shown in Table I and it will be seen

Table I.—Number of medullated fibres in phrenic nerve of cat at different levels. Sections made at 2 or 3 points in the thoracic part of the nerve, the distance between the upper and lower sections varies from 5 to 7 cm.

Nerve.	Upper.	Middle.	Lower.	Difference.
	fibres.	fibres.	fibres.	fibres.
Right phrenic.				
A	709	684	668	41
B	755	—	710	45
C	780	—	739	41
D	840	—	816	24
Left phrenic.				
E	598	579	566	32
F	670	630	623	47

that the upper end has about 40 more fibres than the lower end. The destination of some of these fibres can be seen by staining the fresh tissues of the guinea pig or rat with methylene blue; single fibres or groups of two or three leave the nerve trunk and run sinuously beneath the pleura and over the surface of the vessels (*cf.* fig. 15, A). They appear to end freely and there general arrangement agrees with that of the sensory fibres distributed to the smaller blood vessels, as described by Woollard (1926).

In the dorsal cutaneous nerves where only one or two fibres are giving grouped discharges it is often possible to determine the exact point of origin of the groups by cutting away more and more of the nerve until the point is reached at which the discharge is abolished. An examination of the tissue containing the active point has very often revealed a short length of the nerve trunk with

a single fibre running from it and pursuing a winding course over a small artery in the way described by Woollard. A drawing of one of these preparations is given in fig. 15, B. Whether the nerve fibre must have been cut at some point to activate it is uncertain, for the exposure may be a sufficient stimulus. The reflex movements which occur when a fair-sized nerve is freed from its bed show that the cutting of these scattered fibres is certainly an effective stimulus. On the other hand the terminal part of the fibre seems to be specially prone to persistent activity, for a number of grouped discharges

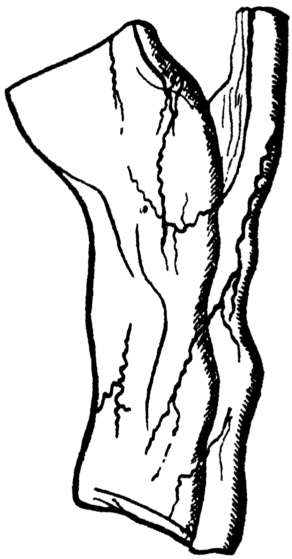

FIG. 15A.

FIG. 15B.

FIG. 15.—A, drawing of methylene blue preparation of phrenic nerve and inferior vena cava (guinea pig). B, drawing of osmic acid preparation of a small portion of a cutaneous nerve with fibres supplying a vessel. A grouped discharge arose from this part of the nerve.

may pass down the nerve when a small length of blood vessel remains attached to one end and all activity ceases when this is cut away. Owing to the ease with which a grouped discharge can be identified it has sometimes been possible to trace one starting from a blood vessel and passing both up and down the nerve trunk. Since the vessels are supplied, in part at least, by side branches from fibres which run on down the nerve, the anti-dromic conduction of the discharge is not surprising.

Another example of persistent activity in sensory fibres from blood vessels is often met with in the rabbit's cardiac depressor. As a rule the discharge does not start unless the cut end is cooled, but it may then suppress all indication

of the volleys which normally pass up from the aorta at each heart beat. This must mean that the discharge is occurring in the fibres which supply the aorta, for otherwise the aortic volleys would be superimposed on it. Moreover Dr. Drury has found that cold Ringer applied to the proxinal end of the cut depressor nerve produces a reflex fall of blood pressure. The fibres in the depressor are of medium-size type with diameters ranging from 3 to 8 μ.

It appears then that the smaller sensory fibres, and particularly those distributed over the vessels, are most likely to give persistent discharges when they are injured, but it is possible that sensory fibres of all sizes may share in the intense activity produced by cutting a large nerve trunk, for all that we can say in such a case is that the motor fibres have little to do with the discharge after the first few seconds.

Discussion.

The practical outcome of this survey is to define the conditions in which a nerve may show a persistent activity which prevents the accurate recording of discharges from particular sense organs and motor neurones. It has been found that the activity is due to repeated excitation from the cut ends of nerve fibres of a particular type, and that it can be abolished or greatly reduced by treating the ends with novocain or by removing the thick nerve sheath and any blood vessels and connective tissue adhering to the nerve. In reflex experiments where a number of large nerve trunks have been cut through it is usual to wait for an hour or more to allow the effects of injury to subside. Since the cut ends are bathed in the tissue fluids and are not irrigated with Ringer, this time is probably enough, but the use of some local anæsthetic might be advantageous, and the method has already been employed by Porter and Hart (1923) in their work on the tenuissimus reflex.

Until more data have been collected it would be unwise to say how far these discharges are responsible for the pain which is felt after injury to the tissues. The fibres in which they occur are distributed to structures which give rise to pain and are not usually credited with giving any other sensation, and the stimuli which produce them—freeing the nerve from surrounding tissues, cutting, etc.—cause reflex movements in the spinal animal and would be likely to cause pain in man. But Erlanger and Gasser (1930) have shown that the dorsal roots and the mixed nerve trunks contain many fibres which conduct at about 1 metre a second and are probably non-medullated, and they agree with Ranson (1921) in considering that these are responsible for protopathic sensation. So far none of the records obtained in this work have shown evidence

of impulses travelling as slowly as 1 metre a second and it may be that the action potentials of such impulses are so small that the sensitivity of the recording system is not great enough to detect them.* A potential change which is less than 1/20 of those recorded in these experiments would certainly pass unnoticed. If these very slow fibres are sensory, they are more likely to be associated with temperature or pain than with any other sensation, and it is true that the amount of activity which can be detected in a nerve supplying exposed tissue often seems inadequate to explain the pain which would be likely to arise from it. Yet it is difficult to believe that these fibres are the only source of pain, for the afferent fibres which are responsible for movements of withdrawal like the flexion reflex must conduct much faster than 1 metre a second. This is shown by many published records of the latency of the flexion reflex and another may be added here.

A digital nerve in the cat's foot was stimulated by single break shocks and the activity of the tibialis anticus was recorded with concentric needle electrodes in the muscle. The stimulus was marked on the record by bringing the stimulating circuit near enough to the amplifier input to produce a small disturbance. The length of afferent nerve, allowing for all possible spread of the stimulus, was 25 cm. and the latent period averaged 0·019 second in seven determinations. If we assume that the whole of this time is occupied in conduction in the afferent nerve, the rate of conduction is 13 metres a second. The true value must be considerably higher.

For the present, however, it is enough to conclude that some of the painful effects produced by injury are caused by discharges of the type shown in these records and it may be suggested that the same kind of reaction may occur in any fibres which give rise to pain.

It will be seen that injury to the nerve fibres is likely to have an intense effect on the central nervous system because of the high frequency of the discharges brought about by the continuous excitation. Matthews has found that the rapid stretching of a muscle will give a very high initial discharge frequency, but the slow rhythms which occur with a weak stimulus show that the sense organ behaves like a structure which regains its full excitability at a much slower rate than a nerve fibre, and the discharges which are normally produced by the sense organs are usually of a much lower frequency than those produced by nerve injury. This is illustrated in fig. 16 (Plate 46) which gives two records of the discharge of a tension receptor in the cat's diaphragm, stretched by the upward pressure of the viscera, and, below, a series of high-

* [*Note added in proof.*—Slow sensory impulses have now been detected in the nerves of the frog. These are produced by irritant substances applied to the skin, but not by mechanical stimulation (Adrian, 'Proc. Physiol. Soc.' (July 5, 1930)).]

frequency discharges produced by cutting a small terminal branch of the nerve in another preparation. It has been argued elsewhere (Adrian 1928) that the essential feature in the pain message is probably not so much a high frequency in the individual fibres as a high concentration of impulses in a particular class of fibre arising within a given area of the central nervous system within a given time. Thus the summed effect of a number of grouped or irregular discharges may have the same effect as a continuous discharge in fewer fibres. But although there are many points which seem to follow from the present results in regard to the mechanism of pain from vessels, fascia and nerve trunks, it will be wiser to leave these over until we know more about the function of the C fibres of Erlanger and Gasser.

The most definite outcome of these observations has been to show that injured mammalian nerve fibres may give a discharge which has many points of resemblance to the discharge of the sense organs. In both the response is rhythmic above a certain frequency, but below this it becomes irregular. For the tension receptors in the frog the critical frequency ranges from 15 to 30 a second (Bronk, 1929, and Matthews, 1929) and in mammalian tension receptors, in spite of the higher temperature, regular discharges may occur at frequencies as low as 35 a second (*cf.* fig. 16, B). For the injured fibre the critical frequency is 150 a second, but the transition from the regular to the irregular sequence bears a close resemblance to the transition in a sense organ discharge, setting aside the difference in time scale. Thus Bronk finds that towards the end of a discharge from the frog's muscle whole groups of impulses tend to drop out of the regular series and in fig. 3 of this paper we find gaps of the same kind in the rhythm from an injured fibre.

Since the discharge of the injured fibre seems to be a consequence of the permanent depolarisation of the injured region we can evidently apply the same kind of explanation to the discharge of the sense organ. In a sense organ which responds to mechanical deformation the stretching of the terminal filaments of the axon may produce a variable degree of depolarisation which will wax and wane with the stimulus and the effect of this would be to produce a rhythmic discharge of varying frequency. Apart from the difference in time relations a parallel case is found in the action of a crystal of $BaCl_2$ on an uninjured nerve fibre. The discharge begins abruptly with a frequency of the order of 150–200 a second and rises within 2–3 seconds to a frequency of 600–800 a second, to fall again more gradually. Here the initial rise is probably due to a partial destruction of the polarised membrane becoming progressively more and more severe, and evidently a partial depolarisation might act in the

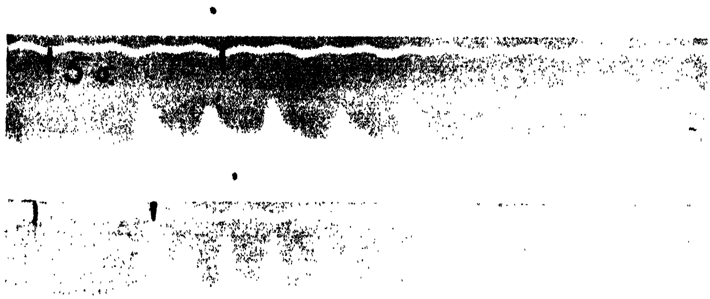

FIG. 9. Grouped discharges from two preparations (dorsal cutaneous nerves) showing a reduction in the size of the second impulse. In the upper record the interval between the first two impulses is 1·9 σ. in the lower 1·25 σ. The shift in the base line of the lower record is due to amplifier distorsion (see p. 597).

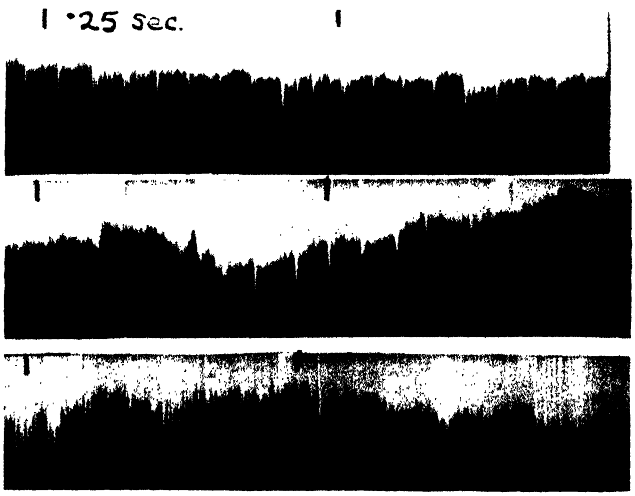

FIG. 16.—Records from uppermost root of phrenic nerve (cat). Cut end treated with novocain. A, lungs deflated and diaphragm stretched by upward pressure of viscera. Regular discharge at 40 a second. A grouped discharge of smaller impulses can also be seen (same preparation as fig. 14, C). B, another experiment. Discharge produced by stretching diaphragm during brief period of deflation of the lungs. Artificial respiration, animal lying on its back; the discharge takes place during expiration. C, another experiment. Discharge produced by cutting branch of phrenic just above diaphragm.

(*Facing p.* 616.)

same way as a submaximal stimulus to an end organ. There is at least no need to regard the action of the end organ as involving processes which differ radically from those which occur in the nerve fibre.

Summary.

(1) The isolated nerves of the frog and tortoise show no activity except during the actual infliction of an injury, but mammalian nerves set up in a warm, moist atmosphere usually give rise to persistent rapid fluctuations of potential which are due to a persistent discharge of impulses in some of the fibres.

(2) Three types of discharge may occur: (*a*) a continuous succession of impulses at a frequency above 150 a second; (*b*) an irregular succession at a lower frequency; (*c*) a grouped discharge, each group consisting of several impulses closely spaced and the groups recurring at a frequency of 5–20 a second. The time relations of these discharges show that the nerve fibre is responding rhythmically to an excitation which outlasts the refractory period of the fibre.

(3) When several fibres are giving discharges of the continuous type, these may become synchronised. The synchronisation is probably due to the stimulating effect of the action currents in one fibre on its neighbour.

(4) The persistent discharges arise from the injured ends of nerve fibres, the permanent depolarisation of the injured region acting as a stimulus to the intact part of the fibre.

(5) The fibres which react in this way to injury are sensory fibres, probably of small diameter. Many of them are distributed to blood vessels and fascia.

(6) The relation of these discharges to pain is discussed. The resemblance between them and the discharges of sense organs shows that the latter are probably caused by a partial depolarisation of the nerve ending, varying with the strength of the stimulus.

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The Iridescent Colours of Birds and Insects.

By Lord RAYLEIGH, For. Sec. R.S.

(Received May 29, 1930.)

(Abstract.)

The reflexion spectra of various brilliantly coloured insects are examined in the ultra-violet. *Morpho* butterflies and *Urania* moths are found to show ultra-violet maxima in general agreement with the theory of interference. In *Morpho achilles* the positions of the ultra-violet maxima indicate that the blue colour is due to a reflexion of the second order. Spectra are reproduced.

Contrary to some previous accounts, no *Morpho* butterflies are found to show their blue colour by transmission. They do show brilliant diffraction spectra by transmission, when suitably mounted in balsam, but these have nothing to do with the blue reflexion.

Iridescent beetles showing a deep red colour at normal incidence may be made to pass through all the colours of the spectrum to violet, provided that arrangements are made to annul refraction at the air-chitin surface so as to

obtain very oblique incidence without. This is in sharp contrast to the surface reflexion of aniline dyes, which do not show much change of colour with incidence.

Some of the golden beetles show transmission spectra of bands corresponding to the reflexion spectra formerly described. These bands vary continuously in position with the part of the specimen examined. It seems impossible to interpret this reasonably except on the theory of interference. The transmission bands of chemical substances do not vary from one sample to another. The spectra are reproduced.

Moist chlorine gas does not destroy the colours of *Morpho* or of *Urania*, though the black background is bleached. Nor does chlorine decolourise the metallic beetles. The colours of all kinds of feathers, however, are rapidly discharged.

Peacock feathers undergo a progressive change of colour in ultra-violet light or long-continued sunlight. Generally speaking, the colours become more refrangible.

Other feathers (*e.g.*, parrot) even when blue are slowly decolourised without change of refrangibility. *Morpho* butterflies and *Urania* also lose colour without change of refrangibility.

Although, *prima facie*, fading under light or chlorine in these cases would seem to favour the idea of a pigment, it is not considered that this view can be maintained. The fading must rather be attributed to the breaking down of an interference structure. It remains unexplained why the progressive change of tint seen in peacock feathers are not seen in the other cases examined.

The generalisation seems to hold good, however, that colours which are stable in chlorine are certainly *not* due to pigments.

[*The full paper is printed in* "Proceedings," *Series A, vol.* 128, *pp.* 624–41, 1930.]

OBITUARY NOTICES.

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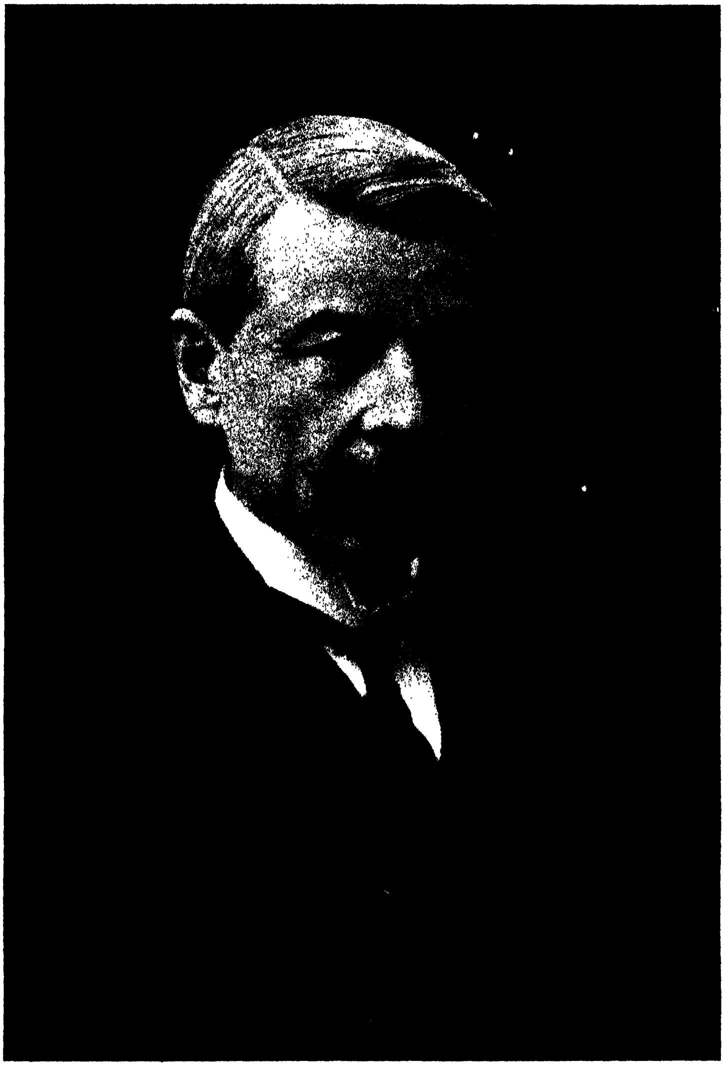


Oldfield Thomas

MICHAEL ROGERS OLDFIELD THOMAS—1858-1929.

OLDFIELD THOMAS was born at Millbrook, Bedfordshire, on February 21, 1858, and was the son of the Rev. J. H. Thomas, who was subsequently appointed Archdeacon of Cape Town. It was here that his son, always known to his family as Oldfield, although his full Christian name was Michael Rogers Oldfield, the last being his mother's maiden surname, first manifested his taste for natural history by collecting insects on the slopes of Table Mountain. On his father's return from the Cape to take up his duties as Vicar of Hillingdon, near Uxbridge, Thomas was sent to Haileybury College; but he gave no promising response to the educational curriculum of the school and, taken away at the age of 18, he secured a clerkship in the office at the British Museum in 1876. Hoping to qualify for an assistantship in the Zoological Department, of which Dr. Albert Günther was then Keeper, he attended during the next two years a course of Huxley's lectures at South Kensington; and in 1878 his hopes were realised by his transference to the zoological staff. His interests at that time were centred in invertebrated animals, and he was filled with dismay when, after a short period of work on Echinoderms, he was informed by Dr. Günther in 1878 of his decision to put him in charge of the mammals. This assistantship he held until his official retirement on February 21, 1923.

From the first he evinced great aptitude for the work required of him as curator of a museum collection; and the early papers he published on his subject soon marked him as the rising systematic mammalogist of the period. Dr. Günther was at that time contemplating the compilation of a catalogue of the Marsupials and Monotremes; but, perceiving Thomas's capacity, he decided to entrust the work to him. A wiser choice could not have been made, for the volume published in 1890 still holds its place as the standard work of reference on the species and genera of these orders, despite the subsequent discovery of a few new forms, like *Notoryctes* and *Cænolestes,* and considerable advance in our knowledge of local races of long-established species. Two valuable papers were also the direct outcome of his examination of the skulls of representatives of these orders, one on the homologies and succession of teeth in the Dasyuridæ, published in the 'Philosophical Transactions,' the other on the teeth of *Ornithorhynchus*, published in the 'Proceedings' of the Royal Society. Similar important papers on mammalian dentition which he wrote later may here be mentioned, namely on the milk teeth of *Orycteropus* and on the number of teeth in the Manatee, the second written in conjunction with his friend the late Richard Lydekker.

While engaged on his catalogue of Marsupials Thomas was compelled to undertake several trips abroad to examine specimens preserved in Leyden, Paris, Genoa. Berlin and elsewhere, and thus made the personal acquaintance of the leading Continental mammalogists of the later Victorian era. Soon after the completion of that volume he embarked upon another great work in combination with Dr. P. L. Sclater. This was 'The Book of Antelopes,' published in four quarto volumes and illustrated with coloured plates depicting all the species of these animals known at the time. Thomas took the responsibility for the descriptions and synonymy of the genera and species of this standard work, which immediately brought him into contact with prominent African sportsmen. But the writing of such books did not appeal to him, and he declined Sir William Flower's invitation to co-operate in the production of the volume on the mammalia which consequently now stands under the joint authorship of Flower and Lydekker. It was at this period that he received the only reward for his work that he ever coveted, namely the Fellowship of the Royal Society, to which he was elected in 1901.

The enduring value of Thomas's Catalogue of Marsupials was due in a great measure to his following the method, practised where possible in those years, of studying mammals by means of alcohol preserved material. But during the years of the nineties zoologists in North America, under the inspiring leadership of Dr. Hart Merriam, struck out a new line of investigation, and, having exhausted the species, in the old sense of the word, of the Continent, started a survey of its mammal fauna from the point of view of geographical distribution and adaptation to environment. By means of intensive collecting, on a scale never dreamt of before, of series of well-prepared skins, measured in the flesh and with skulls attached and labelled with full particulars of sex, date, locality, altitude and other details, a vast amount of interesting information was ascertained, and a wholly unexpected number of previously unnamed local races or sub-species came to light. The reports of the results published by the United States Department of Agriculture at once attracted Thomas's attention, and he was quick to perceive that what was true of the mammalian fauna of North America must be equally true of the faunas of the rest of the world. Instigated by his interest in the subject and by the dominating incentive to enrich the collection under his charge with representatives of as many new mammalian forms as possible, he decided to apply the American system of collecting to South America and all the continents of the Old World so far as the limited resources at his disposal permitted. This decision and the results that followed made a momentous change in the nature of his work, and marked a new epoch in his zoological career. It also gave him, at a time when his health began to fail, an additional interest in life.

This survey of the mammals of the world which he undertook was a colossal and costly task only to be achieved by the co-operation of voluntary workers and by funds far in excess of the share of the Museum purchasing grant that

could fairly be allotted to one class of animals. But, nothing daunted, Thomas soon gathered round him at the Museum a little band of keen naturalists eager to devote their spare time to doing some useful work in the field or to helping in the identification of the collections that were sent in. He got in touch with sportsmen, naturalists, planters and even business men travelling or resident in the British Colonies and Protectorates, and enlisted their services in the cause. This was the work that occupied Thomas's time during the last 30 years of his life. During that period his output of papers, published mostly in the 'Annals and Magazine of Natural History' and in the 'Proceedings of the Zoological Society,' was prodigious, and the number of species or sub-species he designated amounted to about 2000. But in his later years he almost ceased to use nomenclature as a method of expressing kinship, and drifted into the habit, which he defended, of employing the terms "species" or "sub-species" in a geographical instead of a zoological sense. But this idiosyncrasy sinks into insignificance when set against the great enterprise he carried to a successful issue, namely the building up for the national Museum of the finest collection of mammals, taken as a whole, in the world. The magnitude of this achievement, a lasting memorial of his activities, can perhaps only be appreciated by the few still living who remember the collection as it was in the nineties of the last century, and see it, as it is now, stored in huge cabinets filled to overflowing with beautifully made skins of all orders of mammals from all quarters of the globe.

Since I knew Thomas intimately throughout the greater part of his industrious life, perhaps some personal impressions of him, written with as little bias as a close friendship of forty years permits, may be of interest. I joined him as a colleague at the Natural History Museum late in 1885. At that time, when he was verging on 30, he was like the average young Englishman of the period who had been brought up in a well-ordered household of gentle people, had found his level at a public school, learnt the bad taste of self-advertisement and acquired the quality implied by the expression "fair play." But his mind was rather of the practical and methodical than of the intellectual or æsthetic type; and he had profited only to a small extent by the educational system then practised. He had no appreciation of literature, classical or otherwise; and neither then nor subsequently did the perusal of imaginative prose, novels or poetry give him any real pleasure or satisfaction, his reading, such as it was, being restricted mainly to the newspaper, an occasional magazine or volumes of travel. His disposition was eminently sociable. He preferred conversation and argument to books; and aversion to solitude, a curious trait in his character was evinced by his liking for the presence of someone, if only a clerk, in his room when he was hard at work, and by his invariable choice of an occupied carriage when travelling by train. He liked music in a measure; but his

faculties in that direction were not of a high order. In no sense of the word was he artistic. He could neither draw nor paint, nor tell a good picture from a bad one. It cannot even be claimed for him that he was a naturalist in the broadest sense of the word. Beautiful scenery or a night of stars had no special attraction for him, and he was bored by the sea, unless there was shipping to watch, a boat to sail or fish to be caught from a smack. He was uninterested in the geology of the countryside, knew next to nothing of its flora, and very little of its fauna.* As a field naturalist he was rather a collector than an observer. But collecting was, I think, mainly, if not solely, a pleasurable pastime to him when it served the definitely practical purpose of providing for himself or his colleagues specimens which he knew would be useful for work.

I have dwelt at some length upon Thomas's limitations with regard to interests and pursuits because of the important bearing they had upon the latter half of his life. But while still a comparatively young man he was at no loss for recreations. He had an innate proficiency for games, both outdoor and indoor, involving accurate adjustment of eye and hand, like cricket, lawn tennis and billiards. He was also a keen Volunteer in the Artists' Corps, and a very tolerable marksman. Lacrosse and golf he also played for a short time; but the game best suited to his temperament was croquet, which he was happily able to keep up to within six months of his death, spending most of his summer afternoons at Roehampton and devoting his holidays to tournaments at Bournemouth or similar south-coast resorts.

In 1891 he married Mary Kane, the daughter of the late Sir Andrew Clark. She was a charming lady of the Victorian type, a good amateur pianist and artist, with literary tastes and orthodoxly religious, his complete opposite in many ways. Unfortunately they had no family; and his wife's inheritance of a small fortune a few years later placed Thomas in a position of complete independence of the struggle to make both ends meet, to which most scientific men are subjected, thus freeing him from the necessity of supplementing his income by popular writing or lecturing and enabling him to refrain from seeking higher distastefully administrative appointments at the Museum to which his seniority entitled him.

To fame and social advancement Thomas was supremely indifferent. Well aware of his own limitations, he was entirely without conceit, never tried to impress by pretentions of any kind and was generously appreciative of ability in others. He was also mentally alert, gifted with a fund of shrewd commonsense and penetrative ability in reading the characters of his friends. Great determination in carrying through any project that interested him was another

* I learn, however, from Mr. M. A. C. Hinton, who was more intimately associated with Thomas during the last fifteen years of his life than I was, that, when he was about 50, he began to take an interest in trees and in the heavens. A humorous remark he once made about the stars is worth repeating as characteristic of his type of mind:—"What a pity it is we cannot collect them!"

of his attributes, and he would never allow himself to be led by specious arguments into a course of action he did not approve. These qualities made him a valuable member of the Council of the Zoological Society, on which he actively served many years after his first election to it in the last decade of the nineteenth century.

Within a few years of his marriage he began to worry about his health. Troubled by heart palpitations and other symptoms of illness he did not understand, and knowing the physical debility of his family, he gave up all games involving exercise, gradually drifted into the condition of a confirmed invalid, and was so obsessed by the conviction that he had only a few years to live that, when he went to the Argentine for his health, he feared he might never come back to resume his duties at the Museum. This depressing state of mind was fortunately in a measure relieved by a course of treatment which he followed after a consultation with the late Dr. Haig. Of the greatest value to Thomas was the assurance he received that the adoption of a mainly vegetarian diet would at once make a change for the better, and perseverance with it perhaps work a permanent cure. Instilled with this new hope, Thomas entered upon the new dietary *régime* with the tenacity of purpose characteristic of every scheme he embarked upon; and was so impressed by the verification of Haig's prediction that he tried to induce all his friends to follow his example in the matter of food.

It is only fair to point out that his ardent advocacy of this course was based upon the benefit he derived from it, because most of his friends and acquaintances regarded him unjustly, if kindly, as a hypochondriacal crank. Thomas became, it is true, a confirmed valetudinarian, absorbed in the study of his health as affected by diet, reducing himself at times to the verge of starvation, undergoing daily a course of massage and becoming more and more averse to physical exertion of any kind. Probably the distraction and anxieties of children and an interest in literature or art would have prevented the lapse into this deplorable state of mind. Deprived of these, he was hard put to it to find home occupations and, instead of reading, would sit doing some manual work, like knitting, or later in life listening to the wireless. His wife, upon whose company and attentions he was very dependent, unfortunately predeceased him. For a time after her death he carried on his work as before; but as the months went by, he missed her more and more, and finally when he ceased to be interested any longer in his zoological work and showed unmistakable signs of mental derangement, few of his friends were surprised that he died by his own hand in June, 1929.

He left the bulk of his own and his wife's fortune as a collecting fund for the Museum; and shortly before his death he paid for the installation at the Museum of a much-needed lift for the use of the official staff. This was completed and formally opened in May, 1930.

R. I. P.

WALTER BALDWIN SPENCER—1860–1929.

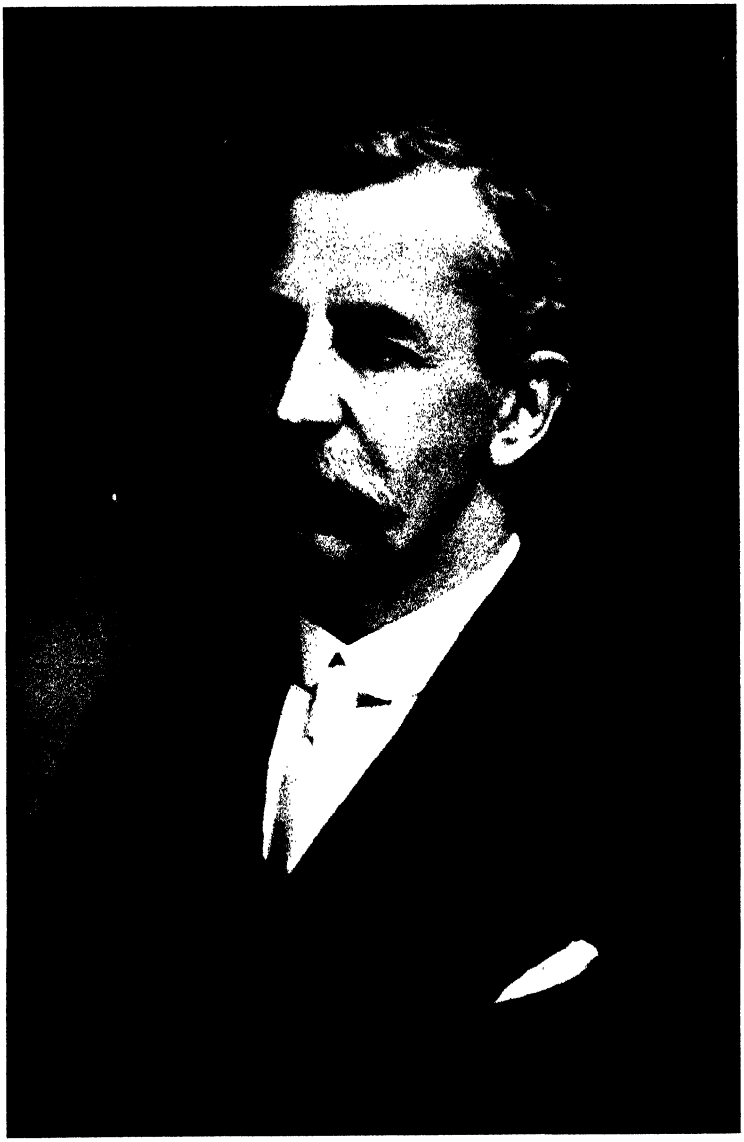
By the death of Sir Baldwin Spencer on July 14, when on his way to embark upon a new field of research in Tierra del Fuego, the Royal Society has lost a Fellow whose name will always be associated with the elucidation of the nature of the pineal eye in *Sphenodon*. He achieved a wider fame by rescuing and recording, just before it would have been too late, the details of the manner of life and thought of the most primitive surviving members of the Human Family.

He was born at Stratford in Lancashire on June 23, 1860. After studying at Owens College, Manchester, he proceeded to Exeter College, Oxford, as a scholar, and subsequently became a Fellow of Lincoln College and a demonstrator of zoology under the Linacre Professor, Henry Nottidge Moseley.

It was Prof. Moseley's practice to make his lectures practical demonstrations of specimens which he described to his class, calling attention to what he could see with the naked eye or with the help of a lens on the actual specimens he was using. On one of these occasions he discussed the well-known white scale on the head of the primitive New Zealand reptile Tuatara (*Sphenodon*), and suggested its possible relationship to the hole in the skull which had long been known, under the name of the parietal foramen, in the crania of fossil and living amphibians and reptiles. Prof. Moseley suggested to Baldwin Spencer, who was then his demonstrator, that he should investigate the relationship of the white scale to the parietal foramen.

Spencer obtained from Prof. Poulton a specimen of the Tuatara, which had been preserved in spirit, and he set to work to investigate it. While he was engaged in this task the Dutch zoologist, Henri W. de Graaf, published in the 'Zoologischer Anzeiger' of March 29, 1886, a paper on the anatomy and development of the epiphysis in amphibia and reptiles. Hence Spencer wrote a letter to 'Nature' (May 13, 1886), giving a preliminary account of his own work. In the following year ('Quarterly Journal of Microscopical Science,' 1887) he published his monograph on the structure of the pineal eye in *Sphenodon* and *Lacerta ocellata*, illustrated by a series of his own beautiful drawings. Discussing the history of the investigation he wrote :—

"Though it was impossible for the external indication of the important organ which forms the subject of [his communication] to escape the notice of naturalists, and more especially of those dealing with the classification of the group, consisting as it does in the modification of a median scale upon the



W. Baldwin Spencer.

dorsal surface of the head, yet it is strange that only within a very recent period had there been any thorough investigation of the structures lying beneath. This is perhaps chiefly to be accounted for by the fact that the structure in question lies usually within the parietal foramen, enclosed tightly by bone and connective tissue, and is thus left intact within the skull on removal either of the skin from the external or the brain from the internal surface."

He emphasised the importance of the work of Leydig in 1872, who seems to have been the first to call attention to the eye-like structure of the pineal appendage. It remained, however, for de Graaf in 1886 first to call attention to the fact that the pineal gland actually becomes modified into a structure comparable to the invertebrate eye. To Spencer belongs the merit of studying its distribution in the animal kindgom and providing a detailed description of the histology of the most complete survival of its structure in *Sphenodon*. His can be regarded as the first adequate description of the morphology of the organ.

Baldwin Spencer's memoir did far more than confirm de Graaf's discovery. The pineal eye in *Sphenodon* reveals a great deal more detail than that of *Anguis*, upon which the Dutch zoologist did his work. Hence he was able to add much fuller data in corroboration of de Graaf's view that the retina of the third vertebrate eye is constructed on a plan entirely different from that of the paired eyes.

After the publication of this famous monograph Baldwin Spencer was appointed Professor of Biology in the University of Melbourne, a Chair which he occupied until 1919, when he was made an emeritus professor. The work of a pioneer in introducing the discipline of modern biology in an Australian University necessarily involved the study of a variety of local animals, both vertebrate and invertebrate, which provided material for a large series of memoirs written during Spencer's first six years in Australia. Then the discovery in Central Australia of a hitherto unknown mammal, the marsupial mole (*Notoryctes*), was responsible for transforming Spencer's career. It impressed upon biologists the need for fuller exploration of the continent. The Horn Scientific Expedition to Central Australia was organised in 1894 and Baldwin Spencer undertook the zoological work. Thus he became acquainted with Mr. F. J. Gillen, who was a Government Inspector in that region, and in the course of many years had acquired an intimate knowledge of the natives.

Spencer became impressed with the exceptional service he might render to anthropology by putting on record the information which Gillen had acquired. His collaboration with the latter was an admirable combination, for Gillen's intimate knowledge of the natives and the fact that he had acquired their confidence made it possible for a skilled scientific observer like Baldwin Spencer, endowed as he was with exceptional literary and artistic gifts,

to rescue important information which otherwise would have been lost for ever.

Hence, after disposing of the work of editing the Reports of the Horn Expedition, Baldwin Spencer returned to Central Australia in 1896 and in co-operation with Gillen undertook the intensive study of the Arunta people. Both investigators were adopted by the natives as fully initiated members of a totemic clan of the Arunta. They were both regarded, for some cryptic reason determined by the older men of the tribe, as belonging to the Witchetty Grub Totem. Hence they were able to witness performances which no other white men had ever seen, or will ever be able to see, because the people they were studying are now virtually extinct. The information acquired during this strange experience was put on record in a volume called "The Native Tribes of Central Australia" (1899).

It is no exaggeration to claim that the publication of this book gave a new orientation to social anthropology. Most of the leading writers upon this subject regarded Spencer and Gillen's description of the customs and practices of the Arunta as a record of the survival of the most primitive type of human culture. Only within recent years has it become possible to shake the firm conviction which grew up during the thirty years after the publication of this book that the customs and beliefs of the Arunta were the prototypes of all primitive behaviour and thought. The late Dr. Rivers showed, however, how profoundly the culture of the natives of Australia had been modified by alien influences.

The value of Baldwin Spencer's work depends, not on the supposition (which is probably erroneous) that the natives of Australia preserve in its entirety a Stone Age culture, but rather on the fact that there have survived with exceptional completeness among them early systems of social organisation and belief, which in large measure are not really primitive but of alien origin. Spencer would never admit this. His merit lies in the thoroughness and the impartiality of his record of the evidence.

The year after the issue of this book Baldwin Spencer was elected to the Fellowship of the Royal Society.

In 1901 he returned to the region north of Alice Springs to renew the study of the Arunta, after which he moved eastwards towards the Gulf of Carpentaria and spent four months living amongst the Warramunga tribe. Thus he gained an insight into the customs and beliefs of the tribes in the northern central area of the continent. The results of this second expedition were published in 1904, in a volume called "The Northern Tribes of Central Australia."

In 1911 the Commonwealth Government had become sufficiently interested in this work to send Baldwin Spencer with a small expedition to the Northern Territory, which enabled him to make his preliminary study of the tribes of the far north, not only on the mainland but also on Bathurst and Melville

Islands. On his return to Melbourne the Commonwealth Government asked him to go back to the Northern Territory and act for one year as Special Commissioner for Aborigines and Chief Protector. He was asked to advise the Government as to the best method of dealing with the aboriginal problem. The results of the work accomplished in the course of these enquiries were published in 1914 in a book called "The Native Tribes of the Northern Territory."

The observations published in these three books, embodying as they do a large mass of accurate and detailed information concerning a most interesting people, had come to play such a large part in the discussion of the social problems of anthropology that Baldwin Spencer was persuaded to return again to Central Australia to clear up a number of doubtful points, and to obtain fresh detail concerning the central problems of Australian ethnology. In 1927 he published perhaps his most important book, "The Arunta," and last year his "Wanderings in Wild Australia."

In 1928, while he was seeing his last book through the press in London, he mentioned his desire to go to Tierra del Fuego and investigate the primitive people living on that desolate island. His friends urged him to continue his work in Australia, for which his qualifications were unique, rather than, at his age, attempt to break fresh ground in the severe climate of Tierra del Fuego, and expose himself to hardships which would tax the strength of much younger men. In spite of this advice he attempted to carry through this new adventure, and in doing so he met his death from an attack of angina when he was travelling by boat in Tierra del Fuego.

Spencer was always keenly interested in art and in his youth had the idea of adopting painting as a profession. He was made a trustee of the Public Library, Art Gallery and Museums of Victoria. He did a great deal to encourage painters in Australia, both by purchasing pictures himself and persuading the Art Gallery in Melbourne to acquire the works of local artists. After he became involved in ethnological research, he was made Director of the National Museum of Victoria.

His greatest achievement was the discovery of Mr. F. J. Gillen and his fruitful collaboration with him in rescuing and putting on record the most complete and the most instructive record of the manner of life, the customs and beliefs, of any primitive people, and of achieving this task just before the information, which he and his collaborator were able to rescue, might have been lost for ever.

He was awarded the C.M.G. in 1904 and created a Knight Commander of the same Order in 1916. He received the Honorary Degree of Doctor of Science from the University of Manchester and was also an Honorary Doctor of Literature, and an Honorary Fellow of Exeter College, Oxford. Spencer was always a most generous man, giving freely to others the material and the information he had collected. The writer of this notice first made Spencer's acquaint-

ance just after he had returned from the Horn Expedition, and received from him some of the most valuable material which had been brought from Central Australia, on giving a promise to study and report on it.

By his death ethnology has lost one of its most accomplished and painstaking field workers, and Australia one of the pioneers who built up the vigorous school of biology on that continent.

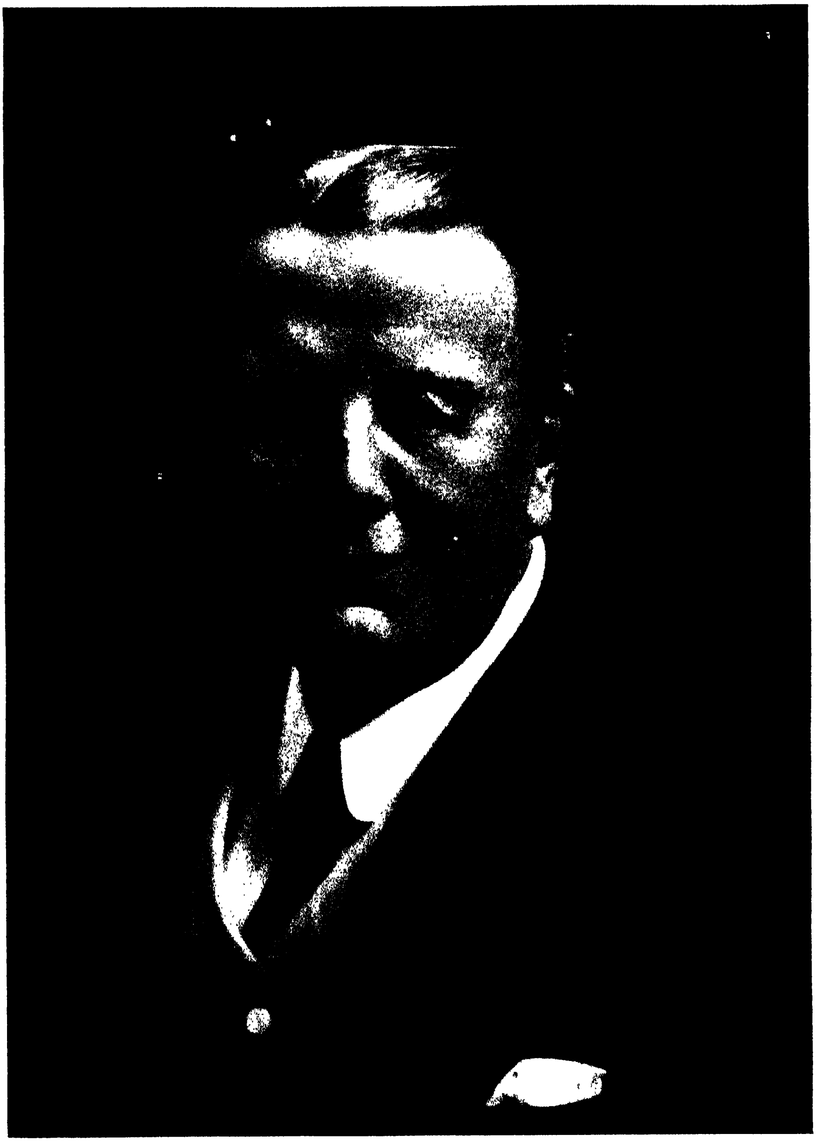
G. E. S.

EDWIN RAY LANKESTER—1847-1929.

Sir Edwin Ray Lankester died on August 15, 1929, the last of a group of brilliant zoologists, which included his friends, F. M. Balfour and H. N. Moseley. Following Darwin and Huxley, they shared in the enthusiasm roused by the publication of the 'Origin of Species,' and by their work and example did much for the advancement of the science of Zoology during the latter half of the nineteenth century. Lankester was a man of exceptional intellectual power; his tall and commanding presence, expressive face, forcible speech, all contributed to make up an impressive personality. Recognised the world over as a great master in science, he was for long the dominating figure among British zoologists, the leader to whom they looked for help and advice.

Edwin Ray Lankester, eldest son of Edwin Lankester, M.D., F.R.S., was born in London in 1847, and educated in the classical tradition at St. Paul's School. His father, who became coroner of Central Middlesex, was a scientific man of distinction interested more especially in microscopy, and a frequent contributor to the pages of the 'Quarterly Journal of Microscopical Science,' of which he was one of the founders and editors. His mother was an accomplished writer. Lankester's boyhood was spent at home in an intellectual and cultivated atmosphere, where he met most of the eminent scientific men of the day—among these were Darwin, Forbes, Hooker, Tyndall, and especially Huxley. From school Lankester went with a scholarship to Downing College, Cambridge; but shortly afterwards, in 1866, migrated to Oxford attracted by the teaching of Rolleston, the first Linacre Professor, who was then initiating his courses of lectures and practical work in the newly-built laboratories of the University Museum. Entering Christ Church as a "Junior Student," he took his degree in Natural Science with first-class honours in 1868, together with his friend Moseley. The Burdett-Coutts scholarship in geology was awarded to him in 1869, and the Radcliffe Travelling Fellowship in 1870.

He then visited Vienna and Leipzig, and studied marine biology in Naples (1871-2). Here he met Anton Dohrn, the founder of the Stazione Zoologica, and in 1874 Balfour and Lankester were among the first to work in that now famous laboratory.



E Ray Lankester

On his return to Oxford in 1872, Lankester was made Fellow and tutor at Exeter College and began his teaching career. First under Rolleston at the Museum and then in his own college he taught until, in 1874, he was elected to the Jodrell chair of Zoology at University College, London. In 1882, however, he was appointed to the professorship of Natural History at Edinburgh; but, finding the conditions there unsuitable, he resigned within a fortnight, and was welcomed back to London, where he resumed the post at University College. This was Lankester's best period, remarkable both for his success as a teacher and for the output of important original researches carried out in his laboratory by himself and his pupils. He delighted to share his knowledge with others, and to rouse in them the interest he felt so deeply. Hence he was a great teacher. His clear and incisive words, his vivid descriptions or lucid explanations fixed the attention and stirred the imagination of his hearers. He delivered inspiring lectures spontaneously, often without notes, trusting to his memory, to the specimens on the table and the diagrams on the wall. He illustrated them by skilfully executed drawings in coloured chalks on the blackboard. With impressive mastery he could marshal the evidence and develop his argument. Perhaps his most precious gift was the power of selecting the essential and discarding the unimportant. In the practical work he took the keenest interest. For Lankester the dissection of an animal was not a matter of dull routine but a voyage of exploration in which new discoveries might be made, the microscope opened up a new and beautiful world full of possible surprises. Carelessness or sloth roused his anger, but to the earnest and enquiring student he gave of his best. Always ready to help or advise colleagues or pupils he took great interest in their work. When consulted he never seemed at a loss for a helpful suggestion or an appropriate comparison drawn from his vast store of well-ordered knowledge. Those who worked with him owed much to his stimulating influence, but he never forced his opinions on them, and allowed them free choice to pursue their own lines of research.

In 1891 he succeeded Moseley at Oxford in the Linacre chair of Comparative Anatomy attached to a Fellowship at Merton College. With his usual energy he soon set to work to carry out improvements in the zoological teaching and accommodation afforded at the Museum, and added a large new building to his Department. He also devoted much attention to the reorganisation of the zoological exhibits, and the methods of displaying museum specimens with an eye to beauty and instruction. Every specimen in the exhibition cases was to be an object of interest and importance and adequately labelled; they were arranged in systematic order and the fossils were included with the recent forms—an important innovation. Of this experience he made good use when in 1898 he was appointed Director of the Natural History Departments of the British Museum and Keeper of Zoology; but was unable to carry out some of his projected reforms owing to the resistence he met from the authorities. He retired from these posts in 1907, at the age of sixty.

Lankester's scientific work extended over almost the whole field of zoology. There is scarcely a group of animals he did not study, scarcely a zoological problem he did not help to solve. His great love of the wonders and beauties of Nature, his insatiable curiosity to know and understand inspired his work, and lasted to the end of his career. He began writing when a mere school-boy, and it is characteristic of the man that he never lost interest in the subjects he took up; his enthusiasm and eagerness for fresh information were not blunted, nor did new interests crowd out old ones from his capacious mind. He was a quick worker and prolific writer—some 170 scientific papers stand to his credit up to the year 1900 in the Royal Society Catalogue. Most of these are quite short, written without waste of words. His first venture into print was a letter on Pteraspis ('Geologist,' 1862), beginning a series of contributions on a remarkable group of fossil fishes, and leading to the important monograph on "The Cephalaspidæ,' published by the Palæontographical Society in 1868–70. In this work, now a recognised classic on the subject, Lankester distinguished the Cephalaspids (Osteostraci) from the Pteraspids (Heterostraci), but cautiously refrained from placing either in any existing group of fishes.

In 1863 he wrote a note on the Gregarinidæ ('Quart. Journ. Micr. Sci.,' vol. 3), the prelude to a series of researches on free-living and parasitic Protozoa that won him a place of honour in the history of Protozoology and Parasitology. The Protozoa had a peculiar fascination for Lankester, who was an expert in microscopy. The credit of having first discovered protozoan blood parasites may be put down to him; since, although the "Undulina" (Trypanosoma) he found in the blood-plasma of the frog (1871) had previously been seen by Brugy, and the "Drepanidium" (Lankesterella) which he described from the blood corpuscles of the frog (1871 and 1882) had probably been recorded by Chaussat, it was Lankester who first recognised as Protozoa these organisms, which had before been considered to be worms. These early researches, together with Laveran's discovery in 1882 of the malarial parasite of human blood, were first steps towards the detailed study of the Hæmoflagellata and Hæmosporidia, which led to most important results during the last decade of the nineteenth century.

Lankester made many contributions on the structure and development of various Invertebrata, more especially the Annelida, Mollusca, and Arthropoda.

In the 'Popular Science Monthly' of 1863 he gave an excellent general account of the little "red worm" Tubifex, shortly after followed by papers on the anatomy of the earthworm ('Quart. Jour. Micr. Sci.,' vol. 4, 1864, and vol. 5, 1865), remarkable productions from so young a writer. Many important observations on budding and zooid formation are recorded in a contribution on the Lower Annelids, Æolosoma and Chætogaster, read before the Linnean Society in 1867 ('Trans. Linn. Soc.,' vol. 26, 1869). Even more important were his studies on the Mollusca, carried out in Oxford, Germany, and Naples.

Of outstanding merit are the "Contributions to the Developmental History of the Mollusca," read in 1874 ('Phil. Trans.,' vol. 165, 1876), in which he traced the development of the shell-gland, the fate of the blastopore, and its distinction from the mouth and anus. The essay which followed on the "Embryology and Classification of the Animal Kingdom, comprising a revision of speculations relative to the origin and significance of the germ layers" ('Quart. Jour. Micr. Sci.,' vol. 16, 1877), contained new and pregnant conclusions, and has had a lasting influence on the science of Embryology.

Believing that clear thinking is helped by a definite nomenclature, Lankester here and elsewhere introduced many new terms which have come into general use, such as archenteron, mesenteron, and blastopore; stomodæum and proctodæum; nephridium, archi-, pro-, meso-, and metanephros. These and later observations led him to the special study of the nature of the body cavities so important for a proper understanding of the general morphology of the higher Metazoa. It is greatly due to him that the true cœlom was distinguished from the blood-vascular system, and that it became established that while in Annelids and Vertebrates the body cavity is cœlomic, in Molluscs and Arthropods it is of a different origin and filled with blood. Finally he was able to give a satisfactory explanation of the unique structure of the arthropod heart ('Quart. Jour. Micr. Sci.,' vol. 34, 1893). Another line of research, initiated in his paper on the appendages and nervous system of Apus ('Quart. Jour. Micr. Sci.,' vol. 21, 1881), helped to elucidate the segmental structure of the head and brain of Arthropods.

The masterly memoir entitled "Limulus an Arachnid" ('Quart. Jour. Micr. Sci.,' vol. 21, 1881) is a triumph of his method. Comparing limb by limb, organ by organ, the king-crab with the scorpion, he proved beyond the possibility of doubt the close affinity to the Arachnids of Limulus hitherto classed with the Crustacea, thereby shedding new light on the whole question of the phylogeny of the Arthropoda. His conclusions were strengthened by later detailed work on the structure of the eyes and other organs in the Arachnida in general.

Among other researches may be mentioned his work on fresh-water Medusæ, on Rhabdopleura, on Amphioxus, and particularly on the development of the atrial cavity of Amphioxus, in conjunction with his pupil A. Willey ('Quart. Journ. Micr. Sci.,' vol. 31, 1890). In later years he wrote on Æluropus, on Lepidosiren, and on Okapia.

On quite other lines were Lankester's pioneer researches on the pigments of animals, which he investigated with the help of the spectroscope. Already in 1867 ('British Association Report') he showed that chlorophyll occurs in Hydra and Spongilla, and hæmoglobin in the muscle of Planorbis, and in the blood of the Chironomus larva and of various Annelids. Later he described new pigments in a number of Invertebrates.

Since his boyhood he had been interested in fossils, more especially those of the Crags of Suffolk. It was therefore with enthusiasm that in later life he took up the question of the existence of Pliocene flint implements in the Red Crag, and wrote a memoir on the subject in the 'Phil. Trans.,' B, vol. 202, 1912.

It was in 1869 that Lankester, who had just graduated at Oxford, became co-editor with his father of the 'Quarterly Journal of Microscopical Science.' Under his able sole editorship from 1878 to 1920 it became the leading British journal of scientific zoology and acquired a world-wide reputation.

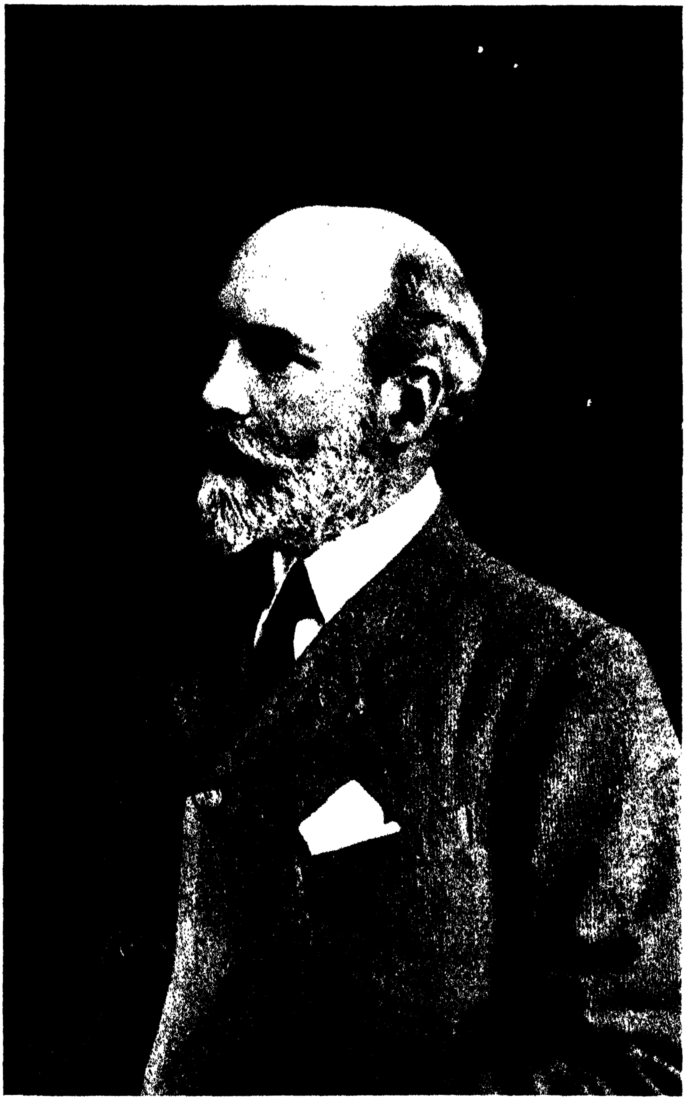
In addition to special papers, Lankester wrote many works of a more general character. The articles he contributed to the 'Encyclopædia Britannica' on Protozoa, Hydrozoa, Mollusca, Arachnida, Polyzoa, and on general zoology, not only contained much that was new and original, but were masterpieces of condensed scientific exposition. Scarcely less remarkable are his general introductions to the volumes of the 'Treatise on Zoology,' of which he was founder and editor. Among his more popular writings may be mentioned 'Comparative Longevity' (1871), 'Degeneration' (1880), 'The Advancement of Science' (1889), 'Extinct Animals' (1905), 'The Kingdom of Man' (1907), 'Science from an Easy Chair' (1910), 'Great Things and Small' (1923).

Sensationalism had no attraction for him, and eager as he was to hear of new discoveries, he was not easily led astray by the extravagant praise of some new theory. For the good work of his predecessors, he had great reverence. The lasting value of Lankester's work depends perhaps most on the soundness of his judgment, combined with penetrating insight and imagination.

Among the greatest services Lankester rendered to zoological science must be reckoned his help in founding the Marine Biological Association and the erection of its Laboratory at Plymouth. For long he took an active interest in its welfare, and the proud position this Laboratory now holds as a centre of biological research is largely due to him.

Lankester was a man of strong feelings, which he did not hesitate to express. Any form of sham, fraud, or injustice roused his anger, and his impetuous temperament sometimes led him into difficulties, and even injured his worldly prospects. He had many interests—artistic, and literary, as well as scientific. He could converse well on almost any subject. His personal charm endeared him to a wide circle of friends at home, and during his frequent visits to the Continent he met almost all the eminent zoologists of his day. Among his intimate friends were Hubrecht, van Beneden, and Metschnikoff, for whose work he had great admiration.

Lankester was elected Fellow of the Royal Society in 1875, was awarded the Royal Medal ten years later, and the Copley Medal in 1913. From the



Walter Heape.

Linnean Society he received the Darwin-Wallace Medal and the Gold Medal. A knighthood was conferred on him on his retirement from the British Museum. He was President of the British Association in 1906, and received many honours from Universities and learned societies at home and abroad.

E. S. G.

WALTER HEAPE—1855-1929.

Walter Heape was the eldest son of Benjamin Heape of Manchester, and Mary Heape, daughter of Joshua Heap of Liverpool. He was born at Polifield, Prestwich, Lancs, on April 25, 1855. He was educated privately until 1872 when he became a student at Owens College, Manchester. He remained there only a year, for in 1873 he began a commercial career with Joseph Heap and Sons, Liverpool; later he was with John Carver Darbishire at Manchester, spending some time at that firm's house at Gibraltar, from which he visited North Africa. From 1875 to 1877 he was with the firm of Richard Harwood and Sons, cotton-spinners, Bolton. He next went on a long voyage to Australia and New Zealand, returning home in 1878. His commercial career was then ended, though he devoted himself to business interests intermittently for a great part of his life.

At precisely what age Heape realised that he had a natural aptitude for scientific pursuits does not appear to be known, but as soon as he returned from the Antipodes he decided definitely to begin his new career. He started by attending Gamgee's laboratory at Owens College, where he learnt some histology. He then went to Oxford to study botany under Lawson, but this only lasted a very short while, for already in 1879 he was established at Cambridge. There he became a student in the laboratories of Foster, Balfour, and Vines. Under Balfour's influence he grew especially interested in embryology, a branch of zoology which at that time, under the influence of the Evolution theory, was undergoing rapid development.

Balfour died in 1882, and the organisation of zoological teaching and research in Cambridge fell to Adam Sedgwick, who enrolled Heape to help him to carry on the school of morphology and embryology which Balfour had founded. For this purpose Heape was appointed University Demonstrator in Morphology. In conjunction with Sedgwick also, Heape undertook the part-authorship of the second and enlarged edition of Foster and Balfour's 'Elements of Embryology,' which for many years was in constant use by zoological students. In 1884 Heape spent the Long Vacation at the Cape, collecting *Peripatus* for Sedgwick and shooting in the Addo Bush. There he shot the elephant whose skeleton he brought home and placed in the Zoological Museum at Cambridge, where it still is.

Heape had no great interest in teaching and he resigned the Demonstratorship in 1885. The same year the Marine Biological Association was started and Heape became, first, Assistant Secretary, and then Superintendent of the Laboratory at Plymouth, a post which he held for two years (1886–88). In the meantime he had visited and worked in Dohrn's laboratory at Naples and the biological laboratory at Woods Holl, U.S.A., besides other fishery establishments. During this period he published some papers, dealing with marine biological and fishery problems, in the *Transactions of the Plymouth Institution* and the *Journal of the Marine Biological Society*. He had previously published an account of the embryology of the mole (*Quart. Jour. Micr. Science*, 1883–6–7), an important investigation which broke new ground and was a model of its kind.

In 1890 Heape was elected to the studentship founded in memory of Balfour for the promotion of zoological research. He was the second Balfour Student (following Caldwell), and he occupied the post for three years, after which he held no further academic appointment.

It was during his tenure of the Balfour Studentship that Heape commenced that series of researches on the comparative physiology of the reproductive processes with which his name will ever be associated. The first of these was upon the menstrual cycle in two species of monkeys, *Macacus rhesus* and *Semnopithecus entellus*, and was published in full in the *Philosophical Transactions* (1894–7). In order to obtain his material and investigate the subject as fully as possible Heape went to India and worked in the Zoological Gardens in Calcutta. As a result of his investigations he was able to describe fully and for the first time the complete series of changes in the histology of the uterine cycle and to correlate these to some extent with the ovarian changes. Among other points of interest he showed that whereas the menstrual cycle recurs throughout the whole year, ovulation and breeding are often restricted to particular seasons, and consequently that menstruation may take place independently of ovulation, a fact which has recently been confirmed by other investigators. Afterwards he supplemented his researches by a study of the menstrual processes in women and published two papers in the *Transactions of the Obstetrical Society* (1894–8). He also published the results of experiments on the successful transplantation of fertilised ova from one rabbit to another (and so into a uterine foster-mother) (1890–3), as well as work on artificial insemination (1897) and on abortion, barrenness and fertility in sheep (1899, *Roy. Soc. Proc.*).

Heape's best known and probably his most important work is his memoir on 'The Sexual Season of Mammals' (*Quart. Jour. Micr. Science*, 1900), which consists of a comparative account of the œstrous cycle for all the different animals for which any data existed. This memoir formed the basis for much future work, and it is noteworthy that the general scheme and terminology formulated by him have become so widely adopted that they are now often

quoted without any reference to the original author. Some of the conclusions reached were only tentative and have had to be revised in the light of later investigation, but the paper must always stand as the first important contribution to the comparative physiology of breeding in the higher animals. It is doubtful whether Heape ever fully grasped the significance of the endocrine activities of the gonads, and he appears at one time to have entertained the view that some of the sexual functions might be discharged after these organs had been removed. But it must not be forgotten that he never had any systematic training in physiology and very little in zoology; yet nevertheless his work gave a remarkable stimulus to research in the physiology of reproduction, and his influence still persists.

In 1905 Heape published a paper (*Roy. Soc. Proc.*) on ovulation and the degeneration of ova in the rabbit, in which he postulated the seasonable appearance in the blood of a substance which he called the "generative ferment" which was responsible for the reproductive and sexual activities. This idea has since been adopted by Mr. John Hammond, and it receives some confirmation from recent work on the secretion of the anterior pituitary body, which appears to play a part similar to that ascribed by Heape to the supposed ferment. To Heape was due the discovery that ovulation in the rabbit is dependent on coition, taking place regularly about ten hours after, an observation which, with some modifications, has since been extended to other animals.

Heape's latest papers were upon sex determination, a subject in which he had for long taken much interest, it being at one time rumoured that he had in certain instances been able to control sex. His views, however, were never clearly formulated, until, under the influence of the Mendelian discoveries, he adopted the theory that the gametes were themselves sexual, and that under certain conditions the male gametes survive and under others the female ones, the environment exercising a selective influence on the numbers of each kind. In support of this view he investigated the proportions of the sexes in canaries, kept under contrasted conditions, as well as in dogs (*Proc. Camb. Phil. Soc.*, 1907), and in the human population of Cuba (*Phil. Trans.*, 1909).

Mention must also be made of Heape's interest in the practical side of his subject, which made a strong appeal to his business instincts. His papers on fertility in animals have already been referred to, but in addition to these he wrote a book on 'The Breeding Industry' (1906), in which he called attention to the great economic importance of animal breeding in Great Britain, and the heavy losses which were annually incurred through failing to apply scientific methods to animal production for commercial purposes. In particular, he stressed the importance of breeders keeping records, so that their experiences should not be lost but be made available to the community. He also showed an active interest in problems of fertility in man and published a book entitled 'Preparation for Marriage' (1914). Another book he wrote was called 'Sex

Antagonism' (1913) and consisted largely of a criticism of Sir James Frazer's 'Totemism and Exogamy.' He also planned the publication of a text-book of comparative embryology, to be completed in three volumes, but of these only the first two have appeared, and he relinquished the editorship after the issue of the first volume, which is on the Invertebrates and was written by Prof. MacBride.

Heape's first official connexion with the Royal Society was in 1896, when he was chosen to serve on the Evolution Committee which was formed with a view to promoting research on all problems relating to evolution. Under its auspices he formulated and conducted a scheme of enquiry into certain matters connected with the breeding of sheep. The investigation was carried out with the co-operation of the Royal Agricultural Society and resulted in the publication of the paper already referred to (*Roy. Soc. Proc.*) as well as one in the *Journal of the Royal Agricultural Society* (1899). Heape was elected a Fellow of the Royal Society in 1906.

In 1885 Heape received from the University of Cambridge the honorary degree of M.A., in recognition of his work and position. The following year he joined Trinity College.

In 1891 he married Ethel, daughter of Joseph Ruston of Lincoln; she died in 1925. They had two sons and one daughter, the eldest son being killed in the great war. After leaving Cambridge in 1907 Heape lived successively in Southwold, London, and Tunbridge Wells, where he died on September 10, 1929.

In his later years, in collaboration with Mr. H. B. Grylls, Heape invented the well-known "Heape and Grylls rapid cinema machine," which was designed for the purpose of photographically recording the movements occurring in high-speed phenomena, such as the bursting of shells, or the penetration of an armour plate by projectiles. The maximum speed at which pictures can be taken by this machine is at the rate of 5000 pictures per second. The invention was completed in 1918. That Heape should have been able to play a leading part in elaborating this remarkable contrivance is further evidence of his versatility and activity of mind.

All who knew Heape well, realised that he was a man of enthusiasms. Moreover, he was ever ready to help and encourage others who were interested in his subject. Had he remained a teacher there is little doubt that he would have founded a school of generative physiology. As it was, his influence lay chiefly in his writings, but through these and the work he accomplished he left an enduring mark.

F. H. A. M.

Harold Wager

HAROLD WAGER—1862–1929.

Harold Wager was born at Stroud in Gloucestershire on March 11, 1862; he died on November 17, 1929, at Hawkswick in Yorkshire. His father—a gardener, descended from a line of ancestors who were small farmers at Painswick, near Stroud—was a man who had the courage of his opinions and confessed himself a follower of Darwin and an admirer of Bradlaugh. Wager's love of Nature, which remained a passion through life, may no doubt be traced to the influence of his father. From his mother, too, the son inherited strength of character and an intellectual outlook.

At the age of thirteen Wager was apprenticed for five years to a local cabinet-maker: his ambition to become a student of science was a controlling force in these early days of uncongenial work. Despite the long hours, from 7 a.m. to 6 p.m., he found time to attend evening classes, and came under the influence of a sympathetic and stimulating teacher, Mr. Paul Smith. He joined a local Natural History Society, to which he contributed several papers. During the period of his apprenticeship he acquired sufficient knowledge of science to be appointed, at the end of the five years, an assistant master at a preparatory school at Reading. At Reading he took full advantage of opportunities for self-education, and eventually gained a scholarship of £50 a year at the Royal College of Science: on this small allowance he lived as a student.

In 1886 on the completion of his College course he became private secretary to Mr. Auberon Herbert, with whom he lived for a year. He met many interesting people, and gained experience which was afterwards of great value to him: he often referred with gratitude to this short interlude in a strenuous life. With the help of the money saved from his salary he was able to return to South Kensington, where he attended lectures by Prof. Huxley, and became a research student under Dr. D. H. Scott, who spoke of him as one of the most promising pupils who had passed through his hands. In 1888 he was appointed Demonstrator in Botany at the Yorkshire College, Leeds, and later Assistant Lecturer. At Leeds he came under the influence of Prof. Miall, whose direct method of teaching Biology and dislike of the conventional practice followed in most Universities, made a deep impression on Wager's receptive mind. His marriage in 1894 to Prof. Miall's only daughter was the beginning of an ideal partnership.

In the winter of 1891–92 he acted as Deputy Reader in Botany at Cambridge. Mr. Francis Darwin, who was then Reader, met Wager at he Cardiff Meeting of the British Association, and being very favourably mpressed by his grasp of Botany, and by the "power of balance" with

which he described his researches and took part in discussions, recommended him for nomination as Deputy Reader. Wager remained at the Yorkshire College until 1894, when he was appointed an Inspector under the Science and Art Department. In 1905 he was transferred as an Inspector to the newly-established Secondary Branch of the Board of Education, and was promoted to a Staff Inspectorship in 1919. For several years he was the Board's representative on the Interdepartmental Committee of the Board of Education and the Ministry of Agriculture. He retired from service under the Board in 1926.

The years that followed his appointment as an Inspector furnish a striking illustration of his devotion to scientific research: though his duties were by no means light, and amply sufficient to fill the working hours of most men, Wager found time to carry on original investigations in many departments of Botany. Habits acquired in early days—the power of concentration, of working under difficulties, and of seizing opportunity remained with him always. He retained throughout life his enthusiasm as a naturalist, always asking questions of Nature and never content until he found answers. He used to say that he could not work well in a University Laboratory—there were too many people about, and the atmosphere was too restless; even the quantity and perfection of the apparatus put him off. He loved to work unconventionally, and made use in turn of his study, an attic, the kitchen, and bathroom for the various kinds of investigation in which he happened to be engaged.

In 1904 Wager was elected Fellow of the Royal Society. In 1905 he was President of Section K at the South African Meeting of the British Association, and for many years he was one of the most useful and welcome members of the Botanical Section. In 1910 he was President of the Mycological Society, and in 1913 President of the Yorkshire Naturalists' Union. In 1915 the University of Leeds conferred upon him the degree of D.Sc. *honoris causa*. Such in brief outline is the life-history of a remarkable man, a man in the fullest sense.

Wager's contributions to Botany cover an unusually wide field. His reputation as an original investigator was established by a series of papers on Fungi: he was the first definitely to prove that the nuclear apparatus of the lower Fungi is in essentials the same as that in the higher plants and animals. He was among the first to employ the microtome in mycological research: conspicuous success led to a general use of his methods. Wager described in full sexual reproduction and the nuclear phenomena of fertilisation and spore-formation in the lower Fungi; also the nuclear changes preceding the formation of spores in some of the higher Fungi. To him we owe the clearest account of the structure of the Yeast cell. His cytological work threw fresh light on the structure of Bacteria and Blue-green Algæ.

His descriptions are concise and lucid: his published work reflects one of the more striking of his qualities—a determination to hold fast to essentials, and an abhorrence of showy superfluities. Wager was an attractive speaker and lecturer, and always spoke with conviction and in language singularly free from all unnecessary technicalities. He was particularly happy in his talks to teachers. Through his frequent lectures to local societies and his attendance at meetings, Wager was brought into contact with almost every serious naturalist, both amateur and professional, in Yorkshire. He was easy of access to the humblest, and there is no doubt that many felt that through him they were in touch with the real source of progress and research—as indeed they were.

A man of his calibre and personal qualities was an immense asset to the Inspectorate. In a Report of a British Association Committee, of which Prof. Miall was Chairman and Wager Secretary, on the Teaching of Botany in Schools he gave much sound advice based on his own experience: he reminded teachers "that to make the most of simple means is an education in itself." He was an all-round naturalist, though, unlike the majority of naturalists, he combined with an intense love of nature a true sense of proportion. He excelled as a microscopist, and in the ingenuity with which he made use of new methods in the investigation of minute structures; but in his attention to details he never allowed his vision to be cramped.

A paper published in 1909 on the Perception of Light in Plants, which is illustrated by photographs of images of objects on the lens-cells of the upper epidermis of leaves, affords a good illustration of his ingenuity in technique, and of his keen desire to test hypothesis by experiment. His conclusion was that "there is no satisfactory evidence to show that the lens-shaped cells (previously described by Haberlandt) can be regarded as special adaptations, either for light perception or for the more efficient illumination of the chlorophyll grains, although it is possible they may be of use for both purposes."

The titles of Wager's papers afford the best illustration of his versatility and breadth of view. Some of his published work suffers from the lack of access to a well-equipped laboratory; but his papers are always stimulating and original. When one considers the conditions under which he worked during the Inspectorship period, one cannot but be amazed at the results achieved.

As an Inspector Wager exerted a far-reaching influence by his personality, his personal contact with schools, his constructive criticism, and his sane commonsense. Impatient of all pretence and self-assertion, he was always ready to help responsive teachers: to those who tried to help themselves he was a sympathetic friend, and never an official to be feared. Teachers without exception spoke of him reverentially and often referred to "inspiration" received from his criticism and suggestions. He was a strong advocate of liberty and independence; teachers, he said, should plan their own courses

and not be hampered by authority. His aim as an Inspector was to make teachers think for themselves, and so fit themselves for making their pupils do the same. He consistently endeavoured to impress upon teachers the notion that Science is not something to be learnt from books; that the starting-point is a desire to know, a habit of asking questions, and a determination to obtain answers. He wrote, in a report on teaching: "how to reconcile liberty with tests of efficiency is a difficult but by no means insoluble problem." Wager successfully solved the problem.

The most unofficial of officials, Harold Wager rendered invaluable service to the Board of Education, and through the Board to National Education; intolerant of sham or humbug, which he never failed to detect, he had a keen sense of humour—an essential qualification for an Inspector of Schools—and the faculty of winning the confidence of teachers, who never looked to him in vain for help and encouragement. Spending little on himself, he was the soul of generosity; by his solidity of character, his readiness to praise where praise was deserved, he won the hearts of those who were privileged to be his friends. Naturalists such as he justify Meredith's dictum, "Men of Science are always the humanest."

Acknowledgment is made to the writers of the Obituary Notices published in *Nature* and the *Journal of Botany* respectively; I am very greatly indebted to Mrs. Wager and to Mr. Morton Wager for supplying some of the most interesting and inspiring biographical facts and generously acceding to my request for permission to publish them.

A. C. S.

WILLIAM TURNER THISELTON-DYER—1843–1928.

With the death of Sir William Turner Thiselton-Dyer (December 23, 1928), who was associated with Kew for thirty years, there passed away one who left a permanent mark on the advance of botany in this country. For Thiselton-Dyer entered on his period of activity at a time when new possibilities were opening up, both in academic botany and in the relation of Kew to the needs of the Empire. It was by seizing these opportunities in both directions that he was able to transform the field and objectives of botany in England, besides raising the Royal Botanic Gardens to a level of pre-eminence and of high efficiency.

As Assistant-Director to Sir Joseph Hooker (1875–1885) and later as Director (1885–1905), the country had in Thiselton-Dyer a far seeing and public-spirited servant who by the use of an acute intelligence was able to remodel the outlook of scientific botany.

His grandfather was William Matthew Thiselton (1783–1842), a printer who became a barrister in order to qualify for public offices. He married Louisa Merzeau, a Frenchwoman descended from a refugee Hugenot family which had established silk looms at Spitalfields. In 1840 Thiselton assumed the surname Dyer by royal license. The second son of his marriage was William George, who became a well-known London physician. He married Miss Firminger, and the subject of the present notice (Sir William Thiselton-Dyer) was born July 28, 1843. There is good evidence that it was his mother who started and fostered ideas of gardening and botany in young Thiselton-Dyer; whilst from the French blood of his grandmother he derived his strongly marked artistic tastes as well, perhaps, as his fondness for the French.

Thiselton-Dyer was educated at King's College School and afterwards (1861) he entered King's College with a view to qualifying in medicine, the profession for which his father intended him. A contemporary at King's (Dr. Sheppard Taylor) has told me that he remembered Dyer "as a man far beyond the average in intelligence, and withal of a most pleasing appearance." Without completing his course, Dyer entered at Christchurch, Oxford, in 1863, as a Junior Student, and graduated in mathematics. His desertion of medicine was the result of his own recognition that he was not robust enough to stand the rough and tumble of a doctor's life.

With his schoolfellow Henry Trimen, who had remained at King's College to qualify in medicine, Thiselton-Dyer made frequent botanical excursions, and jointly they wrote the Flora of Middlesex (1866). At Oxford Thiselton-

Dyer came under the influence of Rolleston and Daubeny, for the latter of whom in particular he retained throughout life the greatest admiration; and it was with regret that in 1913 he found that other commitments prevented him from contributing a chapter on this worthy to "Makers of British Botany," then in preparation.

Among Thiselton-Dyer's Oxford friends reference may be made to H. N. Moseley (of the "Challenger"), and to Ray Lankester who survived Thiselton-Dyer by less than a year. There are many letters from these two young men (Thiselton-Dyer and Lankester) in the early files of 'Nature,' which, as they illustrate the attitude to contemporary science of two notable men, may be consulted with advantage.

On leaving Oxford, Thiselton-Dyer found plenty of openings awaiting him. First he went to the Royal Agricultural College at Cirencester (1868) as Professor of Natural History. Here he had as chemical colleague Prof. A. H. Church (afterwards his neighbour at Kew), and together they edited Johnson's "How Crops Grow," for English use. A year later he took the B.Sc. degree at the University of London, and in 1870 was appointed Professor of Botany in the Royal College of Science, Dublin. Two years later he accepted the chance of returning to London which the professorship of botany to the Royal Horticultural Society gave him, and was soon in touch with Kew, where he acted in a secretarial capacity to Sir Joseph Hooker, the Director, who was already approaching the age of sixty. Hooker was quick to detect Thiselton-Dyer's fine administrative qualities and leant more and more on him, obtaining for him the definitive appointment of Assistant-Director in 1875.

It was during these early years that Thiselton-Dyer joined Huxley at the Royal College of Science as Demonstrator, and designed and conducted the practical botanical courses in biology. This was the first time practical botany had been developed as a laboratory subject, and the effort was far-reaching as it formed the starting point of a scientific treatment in this country. By this means, for instance, were the great results in morphology practically demonstrated and a school of young men trained to spread the good news. Meanwhile, in conjunction with A. W. Bennett, Thiselton-Dyer produced an English edition of Sachs's "Botany," thus rendering accessible, between the covers of a single volume, modern botany as developed by the great German School of Hofmeister, de Bary, Cohn and Sachs himself. England had clung too long to the purely systematic side of botany and the times were ripe for the revolution which Thiselton-Dyer's acute intelligence had engineered.

Having come to anchor at Kew Thiselton-Dyer spent the whole of his active scientific life there. As Assistant-Director he was first entrusted with the colonial activities of the Kew establishment, of which he was quick to see the possibilities. Gradually the whole administrative side fell to his lot, so that in 1885, when he succeeded Sir Joseph Hooker in the Directorship, the change, as he himself described it, was merely a technical one of status.

Here is an extract from an answer to congratulations on his promotion to the Directorship (November 26, 1885):—

> "For my own part I did not desire any change in a position in which I was happy and comfortable. I had quite lost any ambition to lead a larger life. However, now the thing is done I hope you and my colleagues will think it is for the best. To myself the change only seems a technical one, and I hope and believe that the happy terms on which we have all worked together harmoniously for Kew will for many years subsist unbroken."

And this in answer to a similar letter on his knighthood, 14 years later (January 2, 1899):—

> "Thank you for your kind congratulations. My wife and I would have much preferred to remain as we were, but it was not allowed by those in authority. I have long ceased to care for anything but the hope of being of some use while life is spared. I think I have been, but did not want more recognition than I had. However, the staff is pleased and think it good for Kew, so we must make the best of it."

As already indicated, Dyer first made his mark as a teacher. The courses which he arranged set the standard for the whole country; his methods were adopted by the various universities—the much abused University of London, in its old "unregenerate" days, was a pioneer, and by its examination standards gave a lead to the country in the only way it could. Through this work Thiselton-Dyer attracted a group of young men who, when they had proved their ability, were found independent positions. Among the earlier names may be mentioned F. O. Bower, Marshall Ward and Walter Gardiner. Later on (1891) he had the happy inspiration to appoint D. H. Scott to the Honorary Keepership of the Jodrell Laboratory. Through this means a constant flow of young botanical blood passed through Kew. Thiselton-Dyer's interest in and consideration for younger men is gratefully remembered by many.

In administration Thiselton-Dyer's task was harder than that of his predecessors. They laid the foundations on which Thiselton-Dyer had to build, adapting the structure to modern requirements and to the ideals by which he was inspired. To understand the debt of Kew and the country to Thiselton-Dyer, it has to be remembered that the Hookers, father and son, were essentially the founders of Kew, who laid down its material features—the gardens, arboretum, museums, herbarium, and library—the last three being based on the Hookerian collections. The richness of the collections, combined with the eminence and personality of the Hookers, made Kew a mecca for the systematists of all countries, and they were to be found settled on Kew Green during the summer months, consulting the collections to which were att[illegible] Daniel

Oliver and J. G. Baker, to mention no others. Hooker, in conjunction with Bentham, was engaged on the heavy task of preparing the Genera Plantarum and much besides, whilst on top of these distractions and his regular official work he had become President of the Royal Society. When Thiselton-Dyer came to London Hooker pounced on him, gradually allotting him increased powers till the assistant directorship was conferred on him in 1875. Thiselton-Dyer at once grasped the possibilities of the imperial functions of Kew and placed them in the forefront. He saw to it that the botanical establishments of the Empire were connected with Kew by constant interchange of letters, and was a copious and inspiring correspondent—indefatigable in stimulating local effort in botanical exploration and collection, in the exchange of useful plants and the introduction of new cultivations overseas. It must have been with singular satisfaction that he transmitted the first Hevea seedlings from Kew to the East (1875), though the success that was to overtake the venture would take years to mature. Later, when Director, he founded the "Kew Bulletin" as a medium for diffusing information bearing on the exploitation of plants.

Bit by bit Thiselton-Dyer worked over every element in administration; nothing was too small or trifling to escape his observant eye. He increased enormously the amenities available to the young gardeners, organised systematic courses of lectures, and library and reading-room facilities, and having provided them he saw to it that they were used. He would, for instance, count the number of hats on the pegs at the prescribed periods when the library and reading room were accessible, and round up the defaulters. Thiselton-Dyer attached importance to smartness in his subordinates, and succeeded in persuading the gardeners to adopt a uniform of blue serge, supplied locally at a moderate charge. Always well-tailored himself, he gave this matter personal attention and acted as Commandant of the Constables. The latter were mostly ex-soldiers whom he saw degenerating for want of their accustomed discipline; for these men regular parades were held, with a manifest rise in efficiency. All this he did from a sense of duty. Thiselton-Dyer was tidy and methodical in his ways, almost to a fault, and exacted the same from others. He was apt, when colleagues were away on summer leave, to seize the opportunity for a clean up and re-organisation. To some of his conservative colleagues, with strong views of their own, this must have proved a sore trial. Some of his letters, written to prepare them for what they would find on their return, are masterpieces in their way.

Then in the eighties, before Scott had become Keeper, Thiselton-Dyer had a way of dropping into the Jodrell Laboratory to talk to the workers about their investigations—visits which were very welcome. Occasionally, however, they took the less welcome form of "surprise visits," when he would interrogate us as to what we intended to do with certain cherished bottles of pickled material on our shelves. At a place like Kew it was very tempting

to hoard odds and ends, off the line of the moment, for possible future examination—and woe betided the unfortunate wight unable to produce convincing, impromptu justification for such accumulations. They were ruthlessly weeded out and thrown away. This sort of warfare Thiselton-Dyer waged impartially everywhere.

As regards the Gardens, a Director has largely to accept what he inherits. Nevertheless Thiselton-Dyer succeeded in creating new features such as the rock garden and the rose garden ; whilst he annexed, as they fell in, the Queen's Cottage grounds (which he treated as a semi-wild woodland), those of the Palace as well as, under certain restrictions, the Palace itself.

In a different field was the rebuilding, one by one, of the greenhouses, substituting steel construction and modern heating and ventilating for outworn methods. He also completed the Temperate House, an ambitious construction that had remained unfinished for nearly a generation. It was with an almost fiendish delight that he abolished an early heresy by reglazing the tropical fernery with clear white glass in place of green, and great was his triumph at discovering the improved response of the plants under the new regime.

By the time that Thiselton-Dyer came into power the results of industrious tree-planting under previous Directors had become an embarrassment. Everywhere glades, vistas and open spaces had to be carved out of the jungle—a congenial task, as Thiselton-Dyer had marked artistic leanings, and the practical eye of a landscape gardener. Much of the charm of present-day Kew derives from his wise use of the axe.

During his period of office Kew, if ruled by an autocrat, was in the hands of a man of the widest outlook, to whom no personal sacrifice was too great if in its interests. He had no other thought than the advancement of Kew, and through it of botany. Almost every appointment in botany (academic or applied) was in his hands, and, on the whole, his selections have justified themselves. When he was convinced of the wisdom of an object, no personal consideration could stand in his way. If the old Duke of Cambridge took riding exercise in the Gardens, he was to keep off the newly-rolled gravel paths, and, if he transgressed, he would hear from the Director. He was well aware that people were liable to be ruffled, and the following extract from a letter (dated April 24, 1903) summarises his views on the matter: "The fact is that it is impossible for a man in my position to avoid sometimes treading on people's toes. This, of course, gives offence to those who only see one side of the question. I believe I have the reputation amongst my staff of being not too amiable but perfectly just, *i.e.*, according to my lights. At any rate, I try for the latter and can't help the former. I can only ask my friends to put the best construction on my actions."

Thiselton-Dyer was always accessible, but he did not suffer fools gladly. If a bore penetrated to his sanctum he would be offered a cigarette, and when

this was three-quarters consumed the Director would rise from his chair and manœuvre his victim to the door, and the interview terminated.

As a worker in botany Thiselton-Dyer became in early life a very competent systematist. In addition to the "Flora of Middlesex," written jointly with Trimen, he worked on the Indian Dipterocarps, and proved a useful editor of the Flora Capensis and of the Flora of Tropical Africa, and, for a period, of the Icones Plantarum and Botanical Magazine. Some minor contributions issued collectively under the title "Morphological Notes," illustrate his handling in another field, whilst his article in the 9th edition of the "Encyclopædia Britannica" on "Plant Distribution" is still the best general account of the subject. It was always his hope (unfulfilled) to monograph the Cycads, and he assembled in the palm stove at Kew a collection of this group of which he was rightly proud. In the field of Physiology Thiselton-Dyer gave the utmost encouragement to investigators like Brown and Morris, and to Schunk, in their work at the Jodrell Laboratory.

Conspicuous personal achievement in the field of research was incompatible with his duty to Kew, which exacted a whole-time devotion. But he had his reward in creating an organism of the highest efficiency, which rendered great and continuous service to the State.

On his retirement from Kew in 1905 Dyer went to live at Witcombe in Gloucestershire. Here he lived as a country gentleman, taking his share in public affairs. But the consolation of his retirement was the opportunity which it gave him to pursue the classical studies which he loved, and especially the subject of ancient plant names. He gave great assistance in the editing of the new edition of "Liddell and Scott," and wrote several critical papers on the names of plants in the classics. In addition, he carried to an advanced stage of preparation a Glossary of Classical Plant Names, which it is to be hoped may be published, for few men, if any, have combined the requisite classical and botanical training which Thiselton-Dyer possessed.

About ten years before his death he took the unusual course of retiring from the Fellowship of the Royal Society. This step was dictated partly by the circumstance that he was no longer engaged actively in science, partly, perhaps, as one of those petty economies which men make in difficult times.

Without question Thiselton-Dyer was a great administrator who would have come to the front in any field. He was a man of charming manners, the best of companions and a good raconteur. He always secured the loyalty and respect of his staff, though he may not have been popular in the ordinary acceptation of the word. When he left Kew a something was manifest which might have been mistaken for a sense of relief. Really I think it was no more than a sign that a great personality had passed from the theatre of its activities, and that those who remained had not yet re-orientated themselves to the change.

Throughout his life Thiselton-Dyer had the companionship and support of

an accomplished wife. Lady Thiselton-Dyer, a daughter of Sir Joseph Hooker, born and bred at Kew, was in fullest sympathy with her husband's activities. She was an admirable artist and illustrated several of his works, besides giving him direct assistance in many other ways. In June, 1927, about 18 months before Sir William's death, they celebrated their golden wedding. As life began to ebb it was remarkable with what vitality he resisted the encroachments of disease, especially as in middle life he was far from robust.

He is survived by Lady Thiselton-Dyer and by his son and daughter.

F. W. O.

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